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Practitioner's Docket No. 58609 (71432) *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	**B. HANSEN et al.**		
Application No.:	**10/776,917**	Group No.:	**Not Yet Assigned**
Filed:	**February 10, 2004**	Examiner:	**Not Yet Assigned**

For: **OLIGOMERIC COMPOUNDS FOR THE MODULATION OF RAS EXPRESSION**

Commissioner for Patents
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Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: **Denmark**

Application Number: **2003-01539**

Filing Date: **October 20, 2003**

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Kongeriget Danmark

Patent application No.: PA 2003 01539

Date of filing: 20 October 2003

Applicant: (Name and address)
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Denmark

Title: Oligomeric compounds for the modulation ras expression.

IPC: -

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Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

19 February 2004

Pia Petersen

PATENT- OG VAREMÆRKESTYRELSEN

Oligomeric compounds for the modulation ras expression

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras, most preferably Ha-ras. In particular, this invention relates to oligomeric compounds and preferred such compounds are oligonucleotides, which are specifically hybridisable with nucleic acids encoding ras. The oligonucleotide compounds have been shown to modulate the expression of ras and pharmaceutical preparations thereof and their use as treatment of cancer diseases are disclosed.

BACKGROUND OF THE INVENTION

The *ras* proto-oncogenes encode a group of plasma membrane-associated G-proteins that bind guanine nucleotides with high affinity and activates several downstream effector proteins including raf-1, PI3-K etc. that are known to activate several distinct signalling cascades that are involved in the regulation of cellular survival, proliferation and differentiation in response to extracellular stimuli such as growth factors or hormones. The "classical" p21 ras family of mammalian proto-oncogenes consisting of Harvey-ras (Ha-ras), Kirsten-ras (Ki-ras) 4a and 4b and Neuroblastoma-ras (N-ras) are the most well known members of the rapidly expanding Ras superfamily of small GTPases. Several *in vitro* (and *in vivo)* studies have demonstrated that the Ras family of proto-oncogenes are involved in the induction of malignant transformation, see for example Chin et al., (1999) Nature 400, 468-472. Consequently, the p21 Ras family are regarded as important targets in development of anticancer drugs and it has been found that the Ras proteins are either over-expressed or mutated (often leading to constitutive active Ras proteins) in approximately 25% of all human cancers. Interestingly, the *ras* gene mutations in most cancer types are frequently limited to only one of the *ras* genes and are dependent on tumour type and tissue. Ha-ras oncogenic activating mutations have been identified at codon 12, 13 and 61. Activating mutations in the Ha-ras gene are mainly restricted to thyroid, kidney, urinary tract and bladder cancer, while Ha-ras over-expression has been

detected primarily in breast and colon cancer. Because of the evidence of ras involvement in cancer development, interruption of the ras pathway has been a major focus for drug development. Efforts have been concentrated on either inhibiting ras maturation and membrane localization or by inhibiting ras protein expression.

Farnesyltransferase inhibitors target the critical post-translational modification step of farnesylation by inhibiting the farnesyltransferase (FTase) enzyme. However, this enzyme is involved in the farnesylation of a wide variety of proteins and thus it is not possible to specifically inhibit Ha-ras farnesylation by the use of FTase inhibitors.

As specific inhibition of ras isoforms at the protein level has proven difficult due to amino acid sequence homology, inhibition of protein expression by specific targeting of ras isoforms at the mRNA level has been attempted using ribozymes, antisense encoding vectors and antisense oligonucleotides.

Several studies have been published showing tumour growth inhibition in xenograft mouse models treated with antisense oligonucleotides targeted to Ha-ras. Gray et al. (1993) Cancer Research 53, 577-580 showed inhibition of tumour growth of oncogenic Ha-ras transformed NIH-3T3 cells pretreated with antisense oligonucleotides targeting an intron in the 5' UTR of the Ha-ras mRNA. Using a similar model, Wickstrom et al. (1997), Oligonucleotides as Therapeutic Agents, Wiley, London, 124-141, showed 80% inhibition of tumour growth of oncogenic Ha-ras transformed NIH-3T3 cells treated by subcutaneous injection of antisense ODN targeting Ha-ras codon 12 mutation.

Schwab et al. (1994) Proceedings of the National Academy of Science 91, 10640-10464 investigated the effect of phosphorothioate oligonucleotides bound to nano-particles on oncogenic Ha-ras transformed cell lines *in vitro* and *in vivo*. Particle-bound antisense oligonucleotides targeting Ha-ras codon 12 mutation showed a 5-fold decrease in tumour growth compared to an inverse sequence control oligonucleotide when administered by intra-tumoral injection.

An antisense phosphorothioate oligo targeted to the AUG start codon of Ha-Ras (ISIS 2503) developed by Isis Pharmaceuticals has shown potent Ha-ras downregulation *in vitro* and tumour growth inhibition in human tumour xenografts *in vivo*. This antisense oligo was selected as the most potent inhibitor of ras mRNA assayed by Northern blot and it was shown to have an IC50 = 45 nM (Bennett et al.(1996) Antisense Therapeutics, Humana Press, Totowa, New Jersey, 13-47).

Interestingly, the anti-tumour effect of the ISIS 2503 Ha-ras antisense oligo in mouse models was not limited to Ha-ras mutated xenografts, but also showed tumour growth inhibition in Ki-ras mutated tumour xenografts(Cowsert (1997) Anti-Cancer Drug Design 12, 359-371).

Modification of ISIS 2503 with second-generation compounds conferring enhanced affinity and nuclease resistance has been shown to significantly improve the antisense effect. Incorporation of 2'-methoxyethyl (MOE) into ISIS 2503 increased the potency (IC50 = 14,7 nM) and the duration of antisense effect *in vitro* (Cowsert (1997) Anti-Cancer Drug Design 12, 359-371). ISIS 2503 is currently in phase I/II clinical trials either alone or in combination with chemotherapeutic agents against a variety of advanced cancers.

Casey-Cunningham et al.(2001) Cancer 92, 1265-1271, reported that in a phase I study of ISIS 2503 in advanced carcinoma, the compound was well tolerated but none of the 23 patients showed either complete or partial response. However, 4 patients had stable disease for 2 months or longer.

The above-mentioned phosphorothioate and MOE antisense compounds, typically between 20 and 25 base pairs, have been described in several patent applications (WO9222651, WO9408003, WO9428720, WO9849349, WO9902732, WO99227723). However, all disclosed compounds are targeted to two sites on Ha-ras, namely the codon 12 mutation or the AUG start codon, which only constitute a very small portion of the whole target. The codon 12 mutation is also targeted by one antisense sequence disclosed

in WO98500540, which is tested with different phosphorothioate contents.

US6117848 discloses a few Ki-ras antisense oligonucleotides based on phosphorothioate chemistry or O'-2-methyl and in US5872242 a few N-ras phosphorothioate oligonucleotides were disclosed.

US5874416 discloses a single 26-mer antisense oligonucleotide targeted to a portion of the 5'-UTR region where all cytosine bases in CG dinucleotide pairs are 5-methylcytosine.

Most of the oligonucleotides currently in clinical trials are based on the phosphorothioate chemistry from 1988, which was the first useful antisense chemistry to be developed. However, as it has become clear in recent years this chemistry has serious shortcomings that limit its clinical use. These include low affinity for their target mRNA, which negatively affects potency and puts restrictions on how small active oligonucleotides can be thus complicating manufacture and increasing treatment costs. Also, their low affinity translate into poor accessibility to the target mRNA thus complicating identification of active compounds. Finally, phosphorothioate oligonucleotides suffer from a range of side effects that narrow their therapeutic window.

To deal with these and other problems much effort has been invested in creating novel analogues with improved properties. As depicted in the scheme 1 below, these include wholly artificial analogues such as PNA and Morpholino and more conventional DNA analogues such as boranosphosphates, N3'-P5'phosphoroamidates and several 2' modified analogues, such as 2'-F, 2'-O-Me, 2'-O-methoxyethyl (MOE) and 2'-O- (3-aminopropyl)(AP). More recently hexitol nucleic acid (HNA), 2'-F-arabino nucleic acid (2'-F-ANA) and D-cyclohexenyl nucleoside (CeNA) have been introduced.

Many of these analogues exhibit improved binding to complementary nucleic acids, improvements in bio-stability or they retain the ability to recruit a cellular enzyme, RNAseH, which is involved in the mode-of-action of many antisense compounds. None

of them, however, combine all of these advantages and in many cases improvements in one of the properties compromise one or more of the other properties. Also, in many cases new complications have been noted which seriously limits the commercial value of some of the analogues. These include low solubility, complex oligomerisation chemistries, very low cellular up-take, incompatibility with other chemistries, etc.

As a result of the shortcomings in the underlying chemistries there is a lack of therapeutically effective oligonucleotides, such as for instance antisense agents that are effective in inhibiting the synthesis of Ha-ras and which can be used in the treatment of different types of cancers. Accordingly, it is a principal object of the present invention to provide novel oligomeric compounds against the *ras* family of proto-oncogenes preferably the Ha-ras which display enhanced performance compared to the contemporary *ras* compounds thus enhancing their usefulness in the treatment of a variety of cancer diseases. As explained in the following this objective is achieved through the utilisation of a chemistry termed LNA (Locked Nucleic Acid).

SUMMARY OF THE INVENTION

The present invention is directed to oligomeric compounds, particularly LNA antisense oligonucleotides, which are targeted to a nucleic acid encoding the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras, most preferably Ha-ras, and which modulate the expression of the ras. Pharmaceutical and other compositions comprising the oligomeric compounds of the invention are also provided. Further provided are methods of modulating the expression of ras in cells or tissues comprising contacting said cells or tissues with one or more of the oligomeric compounds or compositions of the invention. Also disclosed are methods of treating an animal or a human, suspected of having or being prone to a disease or condition, associated with expression of ras by administering a therapeutically or prophylactically effective amount of one or more of the oligomeric compounds or compositions of the invention. Further, methods of using oligomeric compounds for the inhibition of expression of ras and for treatment of diseases associated with these ras are provided. Examples of such diseases are different types of cancer, such as for instance lung, breast, colon, prostate, pancreas, lung, liver,

thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Illustration of the different designs of the invention: Gapmers, Head- and Tailmers and Mixmers of different composition. For the mixmer, the numbers designate the alternate contiguous stretch of DNA, β-D-oxy-LNA or α-L-LNA. In the drawing, the line is DNA, the gray shadow corresponds to α-L-LNA residues and the rectangle is β-D-oxy-LNA.

Figure 2 illustrates potency and specificity of LNA oligomeric compounds in an in vitro system. The LNA 16-mers shows effective down regulation, much better than the phosphorthioate 20-mer. The LNA oligomeric compounds also shows good specificity, compared to the compounds containing 6 mismatches. (The 4% given in italic have a 28S background smear. This leads to an overestimate of the 28S signal intensity. Therefore the %mRNA is put in brackets on the left side and not corrected for the RNA loading (i.e. the 28S signal).

Figure 3 shows tumor growth reduction by the oligomeric compound Cur2524 (LNA-gapmer). It is also shown that the iso-sequential 16-mer phosphorothioate did not have any effect.

Figure 4 illustrates that the 16-mer LNA oligomeric compound is more potent than the benchmark compound, ISIS2503, here called Cur2119, which is a phosphorthioate 20-mer. The in vivo model was 15PC3 tumour growth inhibition in nude mice treated with 1 mg/kg/day of the oligomeric compounds for 14 days administered continuously by Alzet osmotic pumps.

Figure 5 General scheme of the synthesis of oxy-LNA and amino LNA

Figure 6 General scheme of the synthesis of amino-LNA and thio-LNA

Figure 7 SEQ ID No 1 GenBank accession number J00277

Figure 8 shows that the vivo potency of alpha-L-oxy-LNA oligomeric compounds are at least as good as the beta-D-oxy LNA oligomeric compounds in a 15PC3 and a Miapaca tumor nude mice model dosing 1 mg/kg/day and 2.5 mg/kg/day.

Figure 9 shows that the beta-D-oxy LNA oligomeric compounds 2713 and 2700 are potent inhibitors or tumor growth dosing 5 mg/kg/day in a Miapaca and 15PC3 nude mice model.

Figure 10 shows that alpha-L-oxy LNA and beta-D-oxy LNA oligomeric compounds targeting Ha-ras show low toxicity in mice.

DEFINITION

As used herein, the terms "**target nucleic acid**" encompass DNA encoding the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras, most preferably Ha-ras, , RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA.

As used herein, the term "**gene**" means the gene including exons, introns, non-coding 5′and 3′regions and regulatory elements and all currently known variants thereof and any further variants, which may be elucidated.

As used herein, the terms "**oligomeric compound**" refers to an oligonucleotide which can induce a desired therapeutic effect in humans through for example binding by hydrogen bonding to either a target gene "Chimeraplast" and "TFO", to the RNA transcript(s) of the target gene "antisense inhibitors", "siRNA", "ribozymes" and oligozymes" or to the protein(s) encoding by the target gene "aptamer", spiegelmer" or "decoy".

As used herein, the term "**mRNA**" means the presently known mRNA transcript(s) of a targeted gene, and any further transcripts, which may be identified.

As used herein, the term "**modulation**" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

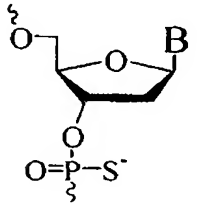
As used herein, the term "**targeting**" an antisense compound to a particular target nucleic acid means providing the antisense oligonucleotide to the cell, animal or human in such a way that the antisense compound are able to bind to and modulate the function of its intended target.

As used herein, "**hybridisation**" means hydrogen bonding, which may be Watson-Crick, Holstein, reversed Holstein hydrogen bonding, etc. between complementary nucleoside or nucleotide bases. Watson and Crick showed approximately fifty years ago that deoxyribo nucleic acid (DNA) is composed of two strands which are held together in a helical configuration by hydrogen bonds formed between opposing complementary nucleobases in the two strands. The four nucleobases, commonly found in DNA are guanine (G), adenine (A), thymine (T) and cytosine (C) of which the G nucleobase pairs with C, and the A nucleobase pairs with T. In RNA the nucleobase thymine is replaced by the nucleobase uracil (U), which similarly to the T nucleobase pairs with A. The chemical groups in the nucleobases that participate in standard duplex formation constitute the Watson-Crick face. Hoogsteen showed a couple of years later that the purine nucleobases (G and A) in addition to their Watson-Crick face have a Hoogsteen face that can be recognised from the outside of a duplex, and used to bind pyrimidine oligonucleotides via hydrogen bonding, thereby forming a triple helix structure.

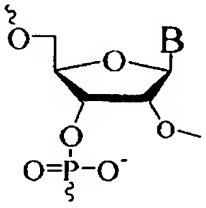
In the context of the present invention "**complementary**" refers to the capacity for precise pairing between two nucleotides or nucleoside sequences with one another. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the corresponding position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The DNA or RNA and the oligonucleotide are considered complementary to each other when a sufficient number of nucleotides in the oligonucleotide can form hydrogen bonds with corresponding nucleotides in the target DNA or RNA to enable the formation of a stable complex. To be stable *in vitro* or *in vivo* the sequence of an antisense compound need not be 100% complementary to its target nucleic acid. The terms "complementary" and "specifically hybridisable" thus imply that

the antisense compound binds sufficiently strongly and specifically to the target molecule to provide the desired interference with the normal function of the target whilst leaving the function of non-target mRNAs unaffected

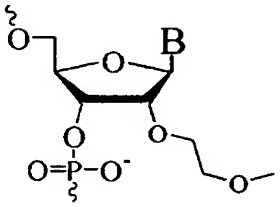
The term "**Nucleic Acid Analogues**" refers to a non-natural nucleic acid binding compound. Nucleic Acid Analogues are described in e.g. Freier & Altmann (Nucl. Acid Res., 1997, 25, 4429-4443) and Uhlmann (Curr. Opinion in Drug & Development (2000, 3(2): 293-213). Scheme 1 illustrates selected examples.



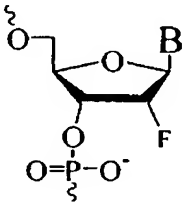
Phosphorthioate



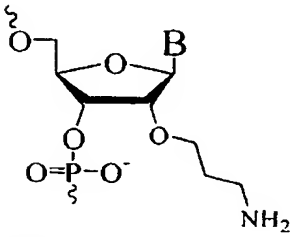
2'-O-Methyl



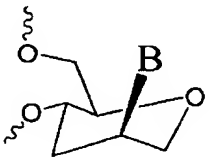
2'-MOE



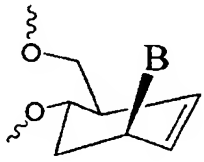
2'-Fluoro



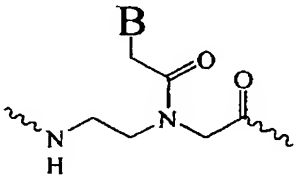
2'-AP



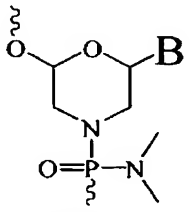
HNA



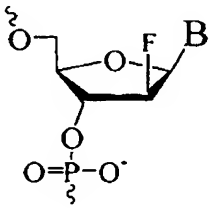
CeNA



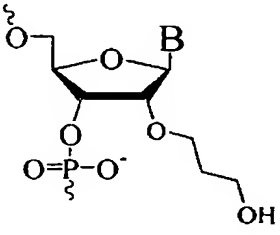
PNA



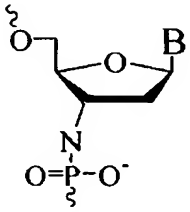
Morpholino



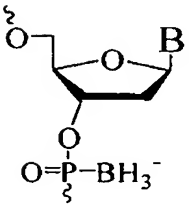
2'-F-ANA



2'-(3-hydroxy)propyl



3'-Phosphoramidate



Boranophosphates

Scheme 1

The term "**LNA**" refers to an oligonucleotide containing one or more bicyclic nucleoside analogues also referred to as a LNA monomer. LNA monomers are described in WO 9914226 and subsequent applications, WO0056746, WO0056748, WO0066604, WO00125248, WO0228875, WO2002094250 and PCT/DK02/00488. One particular example of a thymidine LNA monomer is the (1S,3R, 4R, 7S)-7-hydroxy-1-hydroxymethyl-5-methyl-3-(thymin-1yl)-2,5-dioxa-bicyclo[2:2:1]heptane.

The term "**oligonucleotide**" refers, in the context of the present invention, to an oligomer (also called oligo) or nucleic acid polymer (e.g. ribonucleic acid (RNA) or deoxyribonucleic acid (DNA)) or nucleic acid analogue of those known in the art, preferably Locked Nucleic Acid (LNA), or a mixture thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly or with specific improved functions. A fully or partly modified or substituted oligonucleotides are often preferred over native forms because of several desirable properties of such oligonucleotides such as for instance, the ability to penetrate a cell membrane, good resistance to extra- and intracellular nucleases, high affinity and specificity for the nucleic acid target. The LNA analogue is particularly preferred exhibiting the above-mentioned properties.

By the term "unit" is understood a monomer.

The term "at least one" comprises the integers larger than or equal to 1, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and so forth.

The term "thio-LNA" comprises a locked nucleotide in which at least one of X or Y in Scheme 2 is selected from S or -CH_2-S-. Thio-LNA can be in both beta-D and alpha-L-configuration.

The term "amino-LNA" comprises a locked nucleotide in which at least one of X or Y in Scheme 2 -N(H)-, N(R)-, CH_2-N(H)-, -CH_2-N(R)- where R is selected form hydrogen and C_{1-4}-alkyl. Amino-LNA can be in both beta-D and alpha-L-configuration.

The term "oxy-LNA" comprises a locked nucleotide in which at least one of X or Y in Scheme 2 represents O or -CH_2-O-. Oxy-LNA can be in both beta-D and alpha-L-configuration.

The term "ena-LNA" comprises a locked nucleotide in which Y in Scheme 2 is -CH_2-O-.

By the term "alpha-L-LNA" comprises a locked nucleotide represented as shown in Scheme 3.

By the term "LNA derivatives" comprises all locked nucleotide in Scheme 2 except beta-D-methylene LNA e.g. thio-LNA, amino-LNA, alpha-L-oxy-LNA and ena-LNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding the *ras* family of proto-oncogenes, preferably Ha-ras, Ki-ras and N-ras, most preferably Ha-ras. The modulation is ultimately a change in the amount of ras produced. In one embodiment this is accomplished by providing antisense compounds, which specifically hybridise with nucleic acids encoding Ha-ras. The modulation is preferably an inhibition of the expression of Ha-ras, which leads to a decrease in the number of functional proteins produced.

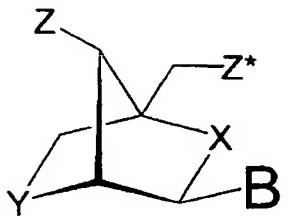
Antisense and other oligomeric compounds of the invention, which modulate expression of the target, are identified through experimentation or though rational design based on sequence information on the target and know-how on how best to design an oligomeric compound against a desired target. The sequences of these compounds are preferred embodiments of the invention. Likewise, the sequence motifs in the target to which these

preferred oligomeric compounds are complementary (referred to as "hot spots") are preferred sites for targeting.

Preferred oligomeric compounds according to the invention are SEQ ID NO 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77 or 79 and their sequences are presented in table 1, 3 and 4. The oligomeric compounds according to the invention are potent modulators of target. For example, in vitro inhibition of target is shown in Table 1 measured by Real time PCR. Figure 2 shows in vitro potency and specificity of oligomeric compounds according to the invention measured by Northern Blot. Very low IC50 of oligomeric compounds is shown in table 2 (compared to the previously reported IC50, see section "Background of the invention"). In vivo specificity and potency of oligomeric compounds are shown in Figure 3. Furthermore, in vivo superiority of a short oligomeric compound compared to a traditional long antisense compound is shown Figure 4. All the above-mentioned experimental observations show that the compounds according to the invention can constitute the active compound in a pharmaceutical composition.

In one embodiment of the invention the oligomeric compounds are containing at least on unit of chemistry termed LNA (Locked Nucleic Acid).

LNA monomer typically refers to a bicyclic nucleoside analogue, as described in the International Patent Application WO 99/14226 and subsequent applications, WO0056746, WO0056748, WO0066604, WO00125248, WO0228875, WO2002094250 and PCT/DK02/00488 all incorporated herein by reference. Preferred LNA monomers structures are exemplified in Scheme 2



Scheme2

X and Y are independently selected among the groups -O-, -S-, -N(H)-, N(R)-, $-CH_2-$ or -CH- (if part of a double bond), $-CH_2-O-$, $-CH_2-S-$, $-CH_2-N(H)-$, $-CH_2-N(R)-$, $-CH_2-CH_2-$ or $-CH_2-CH-$ (if part of a double bond), -CH=CH-, where R is selected form hydrogen and C_{1-4}-alkyl. The asymmetric groups may be found in either orientation.

In Scheme 2 the 4 chiral centers are shown in a fixed configuration. However, also comprised in this invention are compounds of the general Scheme 2 in which the chiral centers are found in different configurations. Thus, each chiral center in Scheme 2 can exist in either R or S configuration. The definition of R (rectus) and S (sinister) are described in the IUPAC 1974 Recommendations, Section E; Fundamental Stereochemistry: The rules can be found in Pure Appl. Chem. 45, 13-30, (1976) and in "Nomenclature of organic Chemistry" pergamon, New York, 1979.

Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group.

The internucleoside linkage may be $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-S-P(O)_2-O-$, $-S-P(O,S)-O-$, $-S-P(S)_2-O-$, $-O-P(O)_2-S-$, $-O-P(O,S)-S-$, $-S-P(O)_2-S-$, $-O-PO(R^H)-O-$, $O-PO(OCH_3)-O-$, $-O-PO(NR^H)-O-$, $-O-PO(OCH_2CH_2S-R)-O-$, $-O-PO(BH_3)-O-$, $-O-PO(NHR^H)-O-$, $-O-P(O)_2-NR^H-$, $-NR^H-P(O)_2-O-$, $-NR^H-CO-O-$, $-NR^H-CO-NR^H-$, $-O-CO-O-$, $-O-CO-NR^H-$, $-NR^H-CO-CH_2-$, $-O-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-$, $-CO-NR^H-CH_2-$, $-CH_2-NR^H-CO-$, $-O-CH_2-CH_2-S-$, $-S-CH_2-CH_2-O-$, $-S-CH_2-CH_2-S-$, $-CH_2-SO_2-CH_2-$, $-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-CO-$, $-CH_2-NCH_3-O-CH_2-$, where R^H is selected form hydrogen and C_{1-4}-alkyl,

The terminal groups are selected independently among from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, C_{1-6}-alkylthio, amino, Prot-N(R^{II})-, Act-N(R^{II})-, mono- or di(C_{1-6}-alkyl)amino, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted C_{2-6}-alkenyloxy, optionally substituted C_{2-6}-alkynyl, optionally substituted C_{2-6}-alkynyloxy, monophosphate, monothiophosphate, diphosphate, dithiophosphate triphosphate, trithiophosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-CH_2-, Act-O-CH_2-, aminomethyl, Prot-N(R^H)-CH_2-, Act-N(R^H)-CH_2-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH(R^H), respectively, Act is an activation group for -OH, -SH, and -NH(R^H), respectively, and R^H is selected from hydrogen and C_{1-6}-alkyl;

The protection groups of hydroxy substituents comprises substituted trityl, such as 4,4'-dimethoxytrityloxy (DMT), 4-monomethoxytrityloxy (MMT), and trityloxy, optionally substituted 9-(9-phenyl)xanthenyloxy (pixyl), optionally substituted methoxytetrahydropyranyloxy (mthp), silyloxy such as trimethylsilyloxy (TMS), triisopropylsilyloxy (TIPS), *tert*-butyldimethylsilyloxy (TBDMS), triethylsilyloxy, and phenyldimethylsilyloxy, *tert*-butylethers, acetals (including two hydroxy groups), acyloxy such as acetyl or halogen substituted acetyls, *e.g.* chloroacetyloxy or fluoroacetyloxy, isobutyryloxy, pivaloyloxy, benzoyloxy and substituted benzoyls, methoxymethyloxy (MOM), benzyl ethers or substituted benzyl ethers such as 2,6-dichlorobenzyloxy (2,6-Cl_2Bzl). Alternatively when Z or Z* is hydroxyl they may be protected by attachment to a solid support optionally through a linker.

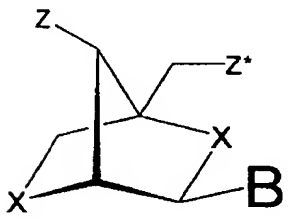
When Z or Z* is amino groups illustrative examples of the amino protection protections are fluorenylmethoxycarbonylamino (Fmoc), *tert*-butyloxycarbonylamino (BOC), trifluoroacetylamino, allyloxycarbonylamino (alloc, AOC), Z benzyloxycarbonylamino (Cbz), substituted benzyloxycarbonylaminos such as 2-chloro benzyloxycarbonylamino (2-ClZ), monomethoxytritylamino (MMT), dimethoxytritylamino (DMT), phthaloylamino, and 9-(9-phenyl)xanthenylamino (pixyl).

In the embodiment above, Act designates an activation group for -OH, -SH, and -NH(R^H), respectively. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphortriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "phosphoramidite" means a group of the formula -P(OR^x)-N(R^y)$_2$, wherein R^x designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, or benzyl, and each of R^y designate optionally substituted alkyl groups, *e.g.* ethyl or isopropyl, or the group -N(R^y)$_2$ forms a morpholino group (-N(CH_2CH_2)$_2$O). R^x preferably designates 2-cyanoethyl and the two R^y are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite.

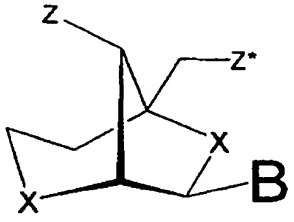
B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 5-propyny-6-fluoroluracil, 5-methylthiazoleuracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 7-propyne-7-deazaadenine, 7-propyne-7-deazaguanine, 2-chloro-6-aminopurine.

Particularly preferred bicyclic structures are shown in Scheme 3 below:

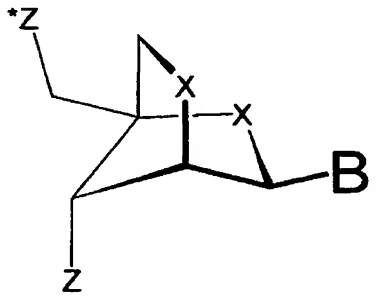


oxy-LNA

when at least one X=O



ena-LNA



alpha-L-LNA

Scheme 3

Where X is -O-, -S-, -NH-, and $N(R^H)$,

Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group.

The internucleotide linkage may be $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-S-P(O)_2-O-$, $-S-P(O,S)-O-$, $-S-P(S)_2-O-$, $-O-P(O)_2-S-$, $-O-P(O,S)-S-$, $-S-P(O)_2-S-$, $-O-PO(R^H)-O-$, $O-PO(OCH_3)-O-$, $-O-PO(NR^H)-O-$, $-O-PO(OCH_2CH_2S-R)-O-$, $-O-PO(BH_3)-O-$, $-O-PO(NHR^H)-O-$, $-O-P(O)_2-NR^H-$, $-NR^H-P(O)_2-O-$, $-NR^H-CO-O-$, where R^H is selected form hydrogen and C_{1-4}-alkyl.

The terminal groups are selected independently among from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, C_{1-6}-alkylthio, amino, Prot-$N(R^H)$-, Act-$N(R^H)$-, mono- or di(C_{1-6}-alkyl)amino, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkyl, optionally substituted monophosphate, monothiophosphate, diphosphate, dithiophosphate triphosphate, trithiophosphate, where Prot is a protection group for -OH, -SH, and $-NH(R^H)$, respectively, Act is an activation group for -OH, -SH, and $-NH(R^H)$, respectively, and R^H is selected from hydrogen and C_{1-6}-alkyl.

The protection groups of hydroxy substituents comprises substituted trityl, such as 4,4'-dimethoxytrityloxy (DMT), 4-monomethoxytrityloxy (MMT), optionally substituted 9-(9-phenyl)xanthenyloxy (pixyl), optionally substituted methoxytetrahydropyranyloxy (mthp), silyloxy such as trimethylsilyloxy (TMS), triisopropylsilyloxy (TIPS), *tert*-butyl-dimethylsilyloxy (TBDMS), triethylsilyloxy, and phenyldimethylsilyloxy, *tert*-butylethers, acetals (including two hydroxy groups), acyloxy such as acetyl Alternatively when Z or Z* is hydroxyl they may be protected by attachment to a solid support optionally through a linker.

When Z or Z* is amino groups illustrative examples of the amino protection protections are fluorenylmethoxycarbonylamino (Fmoc), *tert*-butyloxycarbonylamino (BOC),

trifluoroacetylamino, allyloxycarbonylamino (alloc, AOC), monomethoxytritylamino (MMT), dimethoxytritylamino (DMT), phthaloylamino.

In the embodiment above, Act designates an activation group for -OH, -SH, and -NH(R^H), respectively. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphortriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "phosphoramidite" means a group of the formula -P(OR^x)-N(R^y)$_2$, wherein R^x designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, and each of R^y designate optionally substituted alkyl groups, R^x preferably designates 2-cyanoethyl and the two R^y are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-*O*-(2-cyanoethyl)-phosphoramidite.

B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 2-chloro-6-aminopurine.

Therapeutic principle

A person skilled in the art will appreciate that oligomeric compounds containing LNA can be used to combat ras linked diseases by many different principles, which thus falls within the spirit of the present invention.

For instance, LNA oligomeric compounds may be designed as antisense inhibitors, which are single stranded nucleic acids that prevent the production of a disease causing protein, by intervention at the mRNA level. Also, they may be designed as Ribozymes or Oligozymes which are antisense oligonucleotides which in addition to the target binding domain(s) comprise a catalytic activity that degrades the target mRNA (ribozymes) or

comprise an external guide sequence (EGS) that recruit an endogenous enzyme (RNase P) which degrades the target mRNA (oligozymes)

Equally well, the LNA oligomeric compounds may be designed as siRNA's which are small double stranded RNA molecules that are used by cells to silence specific endogenous or exogenous genes by an as yet poorly understood "antisense-like" mechanism.

LNA oligomeric compounds may also be designed as Aptamers (and a variation thereof, termed Spiegelmers) which are nucleic acids that through intra-molecular hydrogen bonding adopt three-dimensional structures that enable them to bind to and block their biological targets with high affinity and specificity. Also, LNA oligomeric compounds may be designed as Decoys, which are small double-stranded nucleic acids that prevent cellular transcription factors from transactivating their target genes by selectively blocking their DNA binding site.

Furthermore, LNA oligomeric compounds may be designed as Chimeraplasts, which are small single stranded nucleic acids that are able to specifically pair with and alter a target gene sequence. LNA containing oligomeric compounds exploiting this principle therefore may be particularly useful for treating Ha-ras linked diseases that are caused by a mutation in the Ha-ras gene.

Finally, LNA oligomeric compounds may be designed as TFO's (triplex forming oligonucleotides), which are nucleic acids that bind to double stranded DNA and prevent the production of a disease causing protein, by intervention at the RNA transcription level.

Dictated in part by the therapeutic principle by which the oligonucleotide is intended to operate, the LNA oligomeric compounds in accordance with this invention preferably comprise from about 8 to about 60 nucleobases i.e. from about 8 to about 60 linked nucleosides. Particularly preferred compounds are antisense oligonucleotides comprising

from about 12 to about 30 nucleobases and most preferably are antisense compounds comprising about 12-20 nucleobases.

Referring to the above principles by which an LNA oligomeric compound can elicit its therapeutic action the target of the present invention may be the Ha-ras gene, the mRNA or the protein. In the most preferred embodiment the LNA oligomeric compounds is designed as an antisense inhibitor directed against the Ha-ras pre-mRNA or Ha-ras mRNA. The oligonucleotides may hybridize to any site along the Ha-ras pre-mRNA or mRNA such as sites in the 5′ untranslated leader, exons, introns and 3′untranslated tail.

In a preferred embodiment, the oligonucleotide hybridizes to a portion of the human Ha-ras pre-mRNA or mRNA that comprises the translation-initiation site. More preferably, the Ha-ras oligonucleotide comprises a CAT sequence, which is complementary to the AUG initiation sequence of the Ha-ras pre-mRNA or RNA. In another embodiment, the Ha-ras oligonucleotide hybridizes to a portion of the splice donor site of the human Ha-ras pre-mRNA. In yet another embodiment, Ha-ras oligonucleotide hybridizes to a portion of the splice acceptor site of the human Ha-ras pre-mRNA. In another embodiment, the Ha-ras oligonucleotide hybridizes to portions of the human Ha-ras pre-mRNA or mRNA involved in polyadenylation, transport or degradation.

The skilled person will appreciate that preferred oligonucleotides are those that hybridize to a portion of the Ha-ras pre-mRNA or mRNA whose sequence does not commonly occur in transcripts from unrelated genes so as to maintain treatment specificity.

The oligomeric compound of the invention are designed to be sufficiently complementary to the target to provide the desired clinical response e.g. the oligomeric compound must bind with sufficient strength and specificity to its target to give the desired effect. In one embodiment, said compound modulating Ha-ras is designed so as to also modulate other specific nucleic acids which do not encode Ha-ras.

It is preferred that the oligomeric compound according to the invention is designed so that intra- and intermolecular oligonucleotide hybridisation is avoided.

In many cases the identification of an LNA oligomeric compound effective in modulating ras activity in vivo or clinically is based on sequence information on the target gene. However, one of ordinary skill in the art will appreciate that such oligomeric compounds can also be identified by empirical testing. As such Ha-ras oligomeric compounds having, for example, less sequence homology, greater or fewer modified nucleotides, or longer or shorter lengths, compared to those of the preferred embodiments, but which nevertheless demonstrate responses in clinical treatments, are also within the scope of the invention.

Antisense drugs

In one embodiment of the invention the oligomeric compounds are suitable antisense drugs. The design of a potent and safe antisense drug requires the fine-tuning of diverse parameters such as affinity/specificity, stability in biological fluids, cellular uptake, mode of action, pharmacokinetic properties and toxicity.

Affinity & specificity: LNA with an oxymethylene 2'-O, 4'-C linkage (β-D-oxy-LNA), exhibits unprecedented binding properties towards DNA and RNA target sequences. Likewise LNA derivatives, such as amino-, thio- and α-L-oxy-LNA display unprecedented affinities towards complementary RNA and DNA and in the case of thio-LNA the affinity towards RNA is even better than with the β-D-oxy-LNA.

In addition to these remarkable hybridization properties, LNA monomers can be mixed and act cooperatively with DNA and RNA monomers, and with other nucleic acid analogues, such as 2'-O-alkyl modified RNA monomers. As such, the oligonucleotides of the present invention can be composed entirely of β-D-oxy-LNA monomers or it may be composed of β-D-oxy-LNA in any combination with DNA, RNA or contemporary nucleic acid analogues which includes LNA derivatives such as for instance amino-, thio- and α-L-oxy-LNA . The unprecedented binding affinity of LNA towards DNA or RNA target sequences and its ability to mix freely with DNA, RNA and a range of contemporary nucleic acid analogues has a range of important consequences according to the invention for the development of effective and safe antisense compounds.

Firstly, in one embodiment of the invention it enables a considerable shortening of the usual length of an antisense oligo (from 20-25 mers to, *e.g.*, 12-15 mers) without compromising the affinity required for pharmacological activity. As the *intrinsic specificity* of an oligo is inversely correlated to its length, such a shortening will significantly increase the specificity of the antisense compound towards its RNA target. One embodiment of the invention is to, due to the sequence of the humane genome is available and the annotation of its genes rapidly progressing, identify the shortest possible, unique sequences in the target mRNA.

In another embodiment, the use of LNA to reduce the size of oligos significantly eases the process and prize of manufacture thus providing the basis for antisense therapy to become a commercially competitive treatment offer for a diversity of diseases.

In another embodiment, the unprecedented affinity of LNA can be used to substantially enhance the ability of an antisense oligo to hybridize to its target mRNA *in-vivo* thus significantly reducing the time and effort required for identifying an active compound as compared to the situation with other chemistries.

In another embodiment, the unprecedented affinity of LNA is used to enhance the potency of antisense oligonucleotides thus enabling the development of compounds with more favorable therapeutic windows than those currently in clinical trials.

When designed as an antisense inhibitor, the oligonucleotides of the invention bind to the target nucleic acid and modulate the expression of its cognate protein. Preferably, such modulation produces an inhibition of expression of at least 10% or 20% compared to the normal expression level, more preferably at least a 30%, 40%, 50%, 60%, 70%, 80%, or 90% inhibition compared to the normal expression level.

Typically, the LNA oligonucleotides of the invention will contain other residues than β-D-oxy-LNA such as native DNA monomers, RNA monomers, N3′-P5′phosphoroamidates, 2′-F, 2′-O-Me, 2′-O-methoxyethyl (MOE), 2′-O-(3-

aminopropyl) (AP), hexitol nucleic acid (HNA), 2'-F-arabino nucleic acid (2'-F-ANA) and D-cyclohexenyl nucleoside (CeNA). Also, the β-D-oxy-LNA-modified oligonucleotide may also contain other LNA units in addition to or in place of an oxy-LNA group. In particular, preferred additional LNA units include thio-LNA or amino-LNA monomers in either the D-ß or L-α configurations or combinations thereof or ena-LNA. In general, an LNA-modified oligonucleotide will contain at least about 5, 10, 15 or 20 percent LNA units, based on total nucleotides of the oligonucleotide, more typically at least about 20, 25, 30, 40, 50, 60, 70, 80 or 90 percent LNA units, based on total bases of the oligonucleotide.

Stability in biological fluids: One embodiment of the invention includes the incorporation of LNA monomers into a standard DNA or RNA oligonucleotide to increase the stability of the resulting oligomeric compound in biological fluids e.g. through the increase of resistance towards nucleases (endonucleases and exonucleases). The extent of stability will depend on the number of LNA monomers used, their position in the oligonucleotide and the type of LNA monomer used. Compared to DNA and phosphorothioates the following order of ability to stabilize an oligonucleotide against nucleolytic degradation can be established: DNA << phosphorothioates ~ oxy-LNA < α-L-LNA < amino-LNA < thio-LNA.

Given the fact that LNA is compatible with standard DNA synthesis and mixes freely with many contemporary nucleic acid analogues nuclease resistance of LNA- oligomeric compounds can be further enhanced according to the invention by either incorporating other analogues that display increased nuclease stability or by exploiting nuclease-resistant internucleoside linkages e.g. phosphoromonothioate, phosphorodithioate, and methylphosphonate linkages, etc.

Mode of action: Antisense compounds according to the invention may elicit their therapeutic action via a variety of mechanisms and may be able to combine several of these in the same compound. In one scenario, binding of the oligonucleotide to its target

(pre-mRNA or mRNA) acts to prevent binding of other factors (proteins, other nucleic acids, etc.) needed for the proper function of the target i.e. operate by steric hindrance. For instance, the antisense oligonucleotide may bind to sequence motifs in either the pre-mRNA or mRNA that are important for recognition and binding of transacting factors involved in splicing, poly-adenylation, cellular transport, post-transcriptional modifications of nucleosides in the RNA, capping of the 5'-end, translation, etc. In the case of pre-mRNA splicing, the outcome of the interaction between the oligonucleotide and its target may be either suppression of expression of an undesired protein, generation of alternative spliced mRNA encoding a desired protein or both.
In another embodiment, binding of the oligonucleotide to its target disables the translation process by creating a physical block to the ribosomal machinery, i.e. tranlational arrest.

In yet another embodiment, binding of the oligonucleotide to its target interferes with the RNAs ability to adopt secondary and higher order structures that are important for its proper function, i.e. structural interference. For instance, the oligonucleotide may interfere with the formation of stem-loop structures that play crucial roles in different functions, such as providing additional stability to the RNA or adopting essential recognition motifs for different proteins.

In still another embodiment, binding of the oligonucleotide inactivates the target toward further cellular metabolic processes by recruiting cellular enzymes that degrades the mRNA. For instance, the oligonucleotide may comprise a segment of nucleosides that have the ability to recruit ribonuclease H (RNaseH) that degrades the RNA part of a DNA/RNA duplex. Likewise, the oligonucleotide may comprise a segment which recruits double stranded RNAses, such as for instance RNaseIII or it may comprise an external guide sequence (EGS) that recruit an endogenous enzyme (RNase P) which degrades the target mRNA Also, the oligonucleotide may comprise a sequence motif which exhibit RNAse catalytic activity or moieties may be attached to the oligonucleotides which when brought into proximity with the target by the hybridization event disables the target from further metabolic activities.

It has been shown that β-D-oxy-LNA does not support RNaseH activity. However, this can be changed according to the invention by creating chimeric oligonucleotides composed of β-D-oxy-LNA and DNA, called gapmers. A gapmer is based on a central stretch of 4-12 nt DNA or modified monomers recognizable and cleavable by the RNaseH (the gap) typically flanked by 1 to 6 residues of β-D-oxy-LNA (the flanks). The flanks can also be constructed with LNA derivatives. There are other chimeric constructs according to the invention that are able to act via an RNaseH mediated mechanism. A headmer is defined by a contiguous stretch of β-D-oxy-LNA or LNA derivatives at the 5'-end followed by a contiguous stretch of DNA or modified monomers recognizable and cleavable by the RNaseH towards the 3'-end, and a tailmer is defined by a contiguous stretch of DNA or modified monomers recognizable and cleavable by the RNaseH at the 5'-end followed by a contiguous stretch of β-D-oxy-LNA or LNA derivatives towards the 3'-end. Other chimeras according to the invention, called mixmers consisting of an alternate composition of DNA or modified monomers recognizable and cleavable by RNaseH and β-D-oxy-LNA and/or LNA derivatives might also be able to mediate RNaseH binding and cleavage. Since α-L-LNA recruits RNaseH activity to a certain extent, smaller gaps of DNA or modified monomers recognizable and cleavable by the RNaseH for the gapmer construct might be required, and more flexibility in the mixmer construction might be introduced. Figure 1 shows an outline of different designs according to the invention.

Pharmacokinetic properties

The clinical effectiveness of antisense oligonucleotides depends to a significant extent on their pharmacokinetics e.g. absorption, distribution, cellular uptake, metabolism and excretion. In turn these parameters are guided significantly by the underlying chemistry and the size and three-dimensional structure of the oligonucleotide.

As mentioned earlier LNA according to the invention is not a single, but several related chemistries, which although molecularly different all exhibit stunning affinity towards complementary DNA and RNA, Thus, the LNA family of chemistries are uniquely suited

of development oligos according to the invention with tailored pharmacokinetic properties exploiting either the high affinity of LNA to modulate the size of the active compounds or exploiting different LNA chemistries to modulate the exact molecular composition of the active compounds. In the latter case, the use of for instance amino-LNA rather than oxy-LNA will change the overall charge of the oligo and affect uptake and distribution behavior. Likewise the use of thio-LNA instead of oxy-LNA will increase the lipophilicity of the oligonucleotide and thus influence its ability to pass through lipophilic barriers such as for instance the cell membrane.

Modulating the pharmacokinetic properties of an LNA oligonucleotide according to the invention may further be achieved through attachment of a variety of different moieties. For instance, the ability of oligonucleotides to pass the cell membrane may be enhanced by attaching for instance lipid moieties such as a cholesterol moiety, a thioether, an aliphatic chain, a phospholipid or a polyamine to the oligonucleotide. Likewise, uptake of LNA oligonucleotides into cells may be enhanced by conjugating moieties to the oligonucleotide that interacts with molecules in the membrane, which mediates transport into the cytoplasm.

Pharmacodynamic properties

The pharmacodynamic properties can according to the invention be enhanced with groups that improve oligomer uptake, enhance biostability such as enhance oligomer resistance to degradation, and/or increase the specificity and affinity of oligonucleotides hybridisation characteristics with target sequence e.g. a mRNA sequence.

Toxicology

There are basically two types of toxicity associated with antisense oligos: sequence-dependant toxicity, involving the base sequence, and sequence-independent, class-related toxicity. With the exception of the issues related to immunostimulation by native CpG sequence motifs, the toxicities that have been the most prominent in the development of antisense oligonucleotides are independent of the sequence, e.g. related to the chemistry of the oligonucleotide and dose, mode, frequency and duration of administration. The

phosphorothioates class of oligonucleotides have been particularly well characterized and found to elicit a number of adverse effects such as complement activation, prolonged PTT (partial thromboplastin time), thrombocytopenia, hepatotoxicity (elevation of liver enzymes), cardiotoxicity, splenomegaly and hyperplasia of reticuloendothelial cells.

As mentioned earlier, the LNA family of chemistries provide unprecedented affinity, very high bio-stablity and the ability to modulate the exact molecular composition of the oligonucleotide. In one embodiment of the invention, LNA containing compounds enables the development of oligonucleotides which combine high potency with little- if any- phosphorothioate linkages and which are therefore likely to display better efficacy and safety than contemporary antisense compounds.

Manufacture

Oligo- and polynucleotides of the invention may be produced using the polymerisation techniques of nucleic acid chemistry well known to a person of ordinary skill in the art of organic chemistry. Generally, standard oligomerisation cycles of the phosphoramidite approach (S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223) is used, but *e.g.* H-phosphonate chemistry, phosphortriester chemistry can also be used.

For some monomers of the invention longer coupling time, and/or repeated couplings with fresh reagents, and/or use of more concentrated coupling reagents were used.
The phosphoramidites employed coupled with satisfactory >95% step-wise coupling yields. Thiolation of the phosphate is performed by exchanging the normal, *e.g.* iodine/pyridine/H_2O, oxidation used for synthesis of phosphordiester oligomers with an oxidation using Beaucage's reagent (commercially available) other sulfurisation reagents are also comprised. The phosphorthioate LNA oligomers were efficiently synthesised with stepwise coupling yields >= 98 %.

The β-D-amino-LNA, β-D-thio-LNA oligonucleotides, α-L-LNA and β-D-methylamino-LNA oligonucleotides were also efficiently synthesised with step-wise coupling yields ≥ 98% using the phosphoramidite procedures.

Purification of LNA oligomeric compounds was done using disposable reversed phase purification cartridges and/or reversed phase HPLC and/or precipitation from ethanol or butanol. Capillary gel electrophoresis, reversed phase HPLC, MALDI-MS, and ESI-MS was used to verify the purity of the synthesized oligonucleotides. Furthermore, solid support materials having immobilised thereto an optionally nucleobase protected and optionally 5'-OH protected LNA are especially interesting as material for the synthesis of LNA containing oligomeric compounds where an LNA monomer is included in at the 3' end. In this instance, the solid support material is preferable CPG, *e.g.* a readily (commercially) available CPG material or polystyrene onto which a 3'-functionalised, optionally nucleobase protected and optionally 5'-OH protected LNA is linked using the conditions stated by the supplier for that particular material.

Indications

Ha-ras is involved in a number of basic biological mechanisms including red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation and matrix metabolism. For example Ha-ras has been shown to be frequently mutated in bladder, thyroid, kidney carcinoma (Bos (1989), Cancer Research 49: 4682-4689). Over-expression of Ha-ras has been shown in breast and colon carcinoma (P. Horan Hand et al. (1987) Journal of the National Cancer Institute 79: 59-65) The methods of the invention is preferably employed for treatment or prophylaxis against diseases caused by cancer, particularly for treatment of cancer as may occur in tissue such as lung, breast, colon, prostate, pancreas, liver, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, urinary tract or ovaries cancer.

The invention described herein encompasses a method of preventing or treating cancer comprising a therapeutically effective amount of a Ha-ras modulating oligomeric compound, including but not limited to high doses of the oligomer, to a human in need of such therapy. The invention further encompasses the use of a short period of

administration of a Ha-ras modulating oligomeric compound. Normal, non-cancerous cells divide at a frequency characteristic for the particular cell type. When a cell has been transformed into a cancerous state, uncontrolled cell proliferation and reduced cell death results, and therefore, promiscuous cell division or cell growth is a hallmark of a cancerous cell type. Examples of types of cancer, include, but are not limited to, non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia (e.g., acute leukemia such as acute lymphocytic leukemia, acute myelocytic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma), colon carcinoma, rectal carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, cervical cancer, testicular cancer, lung carcinoma, bladder carcinoma, melanoma, head and neck cancer, brain cancer, cancers of unknown primary site, neoplasms, cancers of the peripheral nervous system, cancers of the central nervous system, tumors (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, seminoma, embryonal carcinoma, Wilms' tumor, small cell lung carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, and retinoblastoma), heavy chain disease, metastases, or any disease or disorder characterized by uncontrolled or abnormal cell growth.

Pharmaceutical composition

It should be understood that the invention also relates to a pharmaceutical composition, which comprises a least one antisense oligonucleotide construct of the invention as an active ingredient. It should be understood that the pharmaceutical composition according to the invention optionally comprises a pharmaceutical carrier, and that the pharmaceutical composition optionally comprises further antisense compounds,

chemotherapeutic compounds, anti-inflammatory compounds, antiviral compounds and/or immuno-modulating compounds.

Salts

The oligomeric compound comprised in this invention can be employed in a variety of pharmaceutically acceptable salts. As used herein, the term refers to salts that retain the desired biological activity of the herein identified compounds and exhibit minimal undesired toxicological effects. Non-limiting examples of such salts can be formed with organic amino acid and base addition salts formed with metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with a cation formed from ammonia, *N,N*-dibenzylethylene-diamine, *D*-glucosamine, tetraethylammonium, or ethylenediamine; or (c) combinations of (a) and (b); e.g., a zinc tannate salt or the like.

Prodrugs

In one embodiment of the invention the oligomeric compound may be in the form of a pro-drug. Oligonucleotides are by virtue negatively charged ions. Due to the lipophilic nature of cell membranes the cellular uptake of oligonucleotides are reduced compared to neutral or lipophilic equivalents. This polarity "hindrance" can be avoided by using the pro-drug approach (see e.g. Crooke, R. M. (1998) in Crooke, S. T. *Antisense research and Application*. Springer-Verlag, Berlin, Germany, vol. 131, pp. 103-140). In this approach the oligonucleotides are prepared in a protected manner so that the oligo is neutral when it is administered. These protection groups are designed in such a way that so they can be removed then the oligo is taken up be the cells. Examples of such protection groups are S-acetylthioethyl (SATE) or S-pivaloylthioethyl (*t*-butyl-SATE). These protection groups are nuclease resistant and are selectively removed intracellulary.

Conjugates

In one embodiment of the invention the oligomeric compound is linked to ligands/conjugates. It is way to increase the cellular uptake of antisense oligonucleotides. This conjugation can take place at the terminal positions 5'/3'-OH but the ligands may

also take place at the sugars and/or the bases. In particular, the growth factor to which the antisense oligonucleotide may be conjugated, may comprise transferrin or folate. Transferrin-polylysine-oligonucleotide complexes or folate-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin or folate receptor. Other examples of conjugates/lingands are cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-resistant linkage groups such as phosphoromonothioate, and the like.

The preparation of transferrin complexes as carriers of oligonucleotide uptake into cells is described by Wagner et al ., *Proc. Natl. Acad. Sci. USA* 87, 3410-3414 (1990). Cellular delivery of folate-macromolecule conjugates via folate receptor endocytosis, including delivery of an antisense oligonucleotide, is described by Low et al., U.S. Patent 5,108,921. Also see, Leamon et al., *Proc. Natl. Acad. Sci.* 88, 5572 (1991).

Formulations

The invention also includes the formulation of one or more oligonucleotide compound as disclosed herein. Pharmaceutically acceptable binding agents and adjuvants may comprise part of the formulated drug. Capsules, tablets and pills etc. may contain for example the following compounds: microcrystalline cellulose, gum or gelatin as binders; starch or lactose as excipients; stearates as lubricants; various sweetening or flavouring agents. For capsules the dosage unit may contain a liquid carrier like fatty oils. Likewise coatings of sugar or enteric agents may be part of the dosage unit. The oligonucleotide formulations may also be emulsions of the active pharmaceutical ingredients and a lipid forming a micellular emulsion.

An oligonucleotide of the invention may be mixed with any material that do not impair the desired action, or with material that supplement the desired action. These could include other drugs including other nucleoside compounds.

For parenteral, subcutaneous, intradermal or topical administration the formulation may include a sterile diluent, buffers, regulators of tonicity and antibacterials. The active

compound may be prepared with carriers that protect against degradation or immediate elimination from the body, including implants or microcapsules with controlled release properties. For intravenous administration the preferred carriers are physiological saline or phosphate buffered saline.

Preferably, an oligomeric compound is included in a unit formulation such as in a pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount without causing serious side effects in the treated patient.

Administration

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be (a) oral (b) pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, (c) topical including epidermal, transdermal, ophthalmic and to mucous membranes including vaginal and rectal delivery; or (d) parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. In one embodiment the active oligo is administered IV, IP, orally, topically or as a bolus injection or administered directly in to the target organ.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Compositions and formulations for oral administration include but is not restricted to powders or granules, microparticulates, nanoparticulates, suspensions or solutions in

water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Delivery

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self- emulsifying solids and self-emulsifying semisolids. Delivery of drug to tumour tissue may be enhanced by carrier-mediated delivery including, but not limited to, cationic liposomes, cyclodextrins, porphyrin derivatives, branched chain dendrimers, polyethylenimine polymers, nanoparticles and microspheres (Dass CR. J Pharm Pharmacol 2002; 54(1):3-27).

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels and suppositories. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Combination drug

Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, ibuprofen, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

LNA containing oligomeric compound are useful for a number of therapeutic applications as indicated above. In general, therapeutic methods of the invention include administration of a therapeutically effective amount of an LNA-modified oligonucleotide to a mammal, particularly a human.

In a certain embodiment, the present invention provides pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g. mithramycin and oligonucleotide), sequentially (e.g. mithramycin and oligonucleotide for a period of time followed by another agent and oligonucleotide), or in combination with one or more other such chemotherapeutic agents or in combination with radiotherapy. All chemotherapeutic agents known to a person skilled in the art are here incorporated as combination treatments with compound according to the invention.

Anti-inflammatory drugs, including but not limited to nonsteroidal anti- inflammatory drugs and corticosteroids, antiviral drugs, and immuno-modulating drugs may also be combined in compositions of the invention. Two or more combined compounds may be used together or sequentially.

In another embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially.

Dosage

Dosing is dependent on severity and responsiveness of the disease state to be treated, and the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient.

Optimum dosages may vary depending on the relative potency of individual oligonucleotides. Generally it can be estimated based on EC50s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 1 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 10 years or by continuous infusion for hours up to several months. The repetition rates for dosing can be estimated based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state.

Uses

The LNA containing oligomeric compounds of the present invention can be utilized for as research reagents for diagnostics, therapeutics and prophylaxis . In research, the antisense oligonucleotides may be used to specifically inhibit the synthesis of ras genes in cells and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention. In diagnostics the antisense oligonucleotides may be used to detect and quantitate ras expression in cell and tissues by Northern blotting, in-situ hybridisation or similar techniques. For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of ras is treated by administering antisense compounds in accordance with this invention. Further provided are methods of treating an animal particular mouse and rat and treating a human, suspected of having or being prone to a disease or condition, associated with expression of ras by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

Example 1; MONOMER SYNTHESIS

Preparation of the monomers shown in Scheme 1 in which Y and X are -O- and Z and Z* are protected -O- is described in great detail in the reference, Koshkin et al, *J. Org. Chem.*, **2001**, *66*, 8504-8512; Sørensen et al., J. Am. Chem. Soc., 2002, 124 (10), 2164-2176; Pedersen et al., Synthesis, 2002, 6, 802-809 and references found therein, where the protection groups of Z and Z* are respectively oxy-N,N-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite and dimethoxytrityloxy . The preparation of monomers of the Scheme 1 in which X is –O- and Y is -S- and -N(CH_3)- is described below (see also Figure 5 and 6):

1-(3-*O*-Benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl-*β*-D-*erythro*-pentofuranosyl)thymine (21). Nucleoside **20** (Koshkin et al., J. Org. Chem. 2001, 66, 8504-8512) (30 g, 52 mmol) was dissolved in MeOH (600 mL), and the solution was cooled to 0 °C. Freshly prepared sat. methanolic ammonia (600 mL) was added, and the mixture was allowed to reach rt. After 5 h at rt the reaction was quenched with glacial acetic acid (50 mL) and transferred to a beaker, where it was neutralised with sat. aq $NaHCO_3$. EtOAc (900 mL) and brine (500 mL) was added and the phases were separated. The aq phase was extracted with EtOAc (3 x 500 mL) and the combined organic phases were washed with sat. aq $NaHCO_3$ (500 mL) and brine (500 mL). The organic phase was dried (Na_2SO_4), filtered and the solvent removed in vacuo to afford **21** (27 g, 97%) as a white foam. R_f = 0.33 (100% EtOAc); ESI-MS *m/z* found 557.0 ($[MNa]^+$, calcd 557.1); ^{1}H NMR ($CDCl_3$) δ 10.21 (br s, 1H, NH), 7.33-7.25 (m, 6H, Ph, H6), 5.77 (d, J = 3.9, 1H, H1'), 4.84 (d, J = 11.4, 1H, H3'), 4.59-4.57 (m, 3H), 4.42-4.37 (m, 3H), 4.26-4.19 (m, 2H) (H2', H2", H5", C$\underline{H}_2$Ph, OH), 2.98 (s, 3H, CH_3), 2.76 (s, 3H, CH_3), 1.80 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 162.5 (C4), 151.0 (C2), 136.7 (Ph), 136.2 (C6), 128.5, 128.3, 128.2 (Ph), 111.3 (C5), 92.1 (C1'), 84.0 (C4'), 77.7 (C3'), 74.1, 73.5 (C2', $\underline{C}H_2$Ph), 68.6, 68.3 (C5', C1''), 37.2, 37.1 (Ms), 12.0 (CH_3); Anal. calcd for $C_{20}H_{26}N_2O_{11}S_2$: C, 44.9; H, 4.9; N, 5.2. Found: C, 45.0; H, 4.7; N, 5.1.

1-(3-*O*-Benzyl-2,5-di-*O*-methanesulfonyl-4-*C*-(methanesulfonyloxymethyl)-*β*-D-*erythro*-pentofuranosyl)thymine (13B). Nucleoside **21** (20 g, 37 mmol) was dissolved

in anhyd dichloromethane (100 mL) and anhyd pyridine (100 mL) was added. The solution was cooled to 0 °C and methanesulfonyl chloride (4.4 mL, 56 mmol) was added dropwise. After 2 h the reaction was quenched with sat. aq $NaHCO_3$ (200 mL), and the phases were separated. The aq phase was extracted with dichloromethane (2 x 150 mL), and the combined organic phases were washed with aq HCl (1 M, 2 x 200 mL), sat. aq $NaHCO_3$ (2 x 250 mL) and brine (250 mL). The organic phase was dried (Na_2SO_4), filtered and the solvent was removed in vacuo. The crude product was co-evaporated with toluene affording **13B** (22 g, 96%) as a white foam. R_f = 0.41 (100% EtOAc); ESI-MS *m/z* found 635.0 ($[MNa]^+$, calcd 635.1). All analytical data were identical to those previously reported.(Håkansson, A. E.; Koshkin, A.; Sørensen, M. D.; Wengel, J. *J.Org.Chem.* **2000**, *65*, 5161-5166).

2,2'-Anhydro-1-(3-*O*-benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl-*β*-D-*threo*-pentofuranosyl)thymine (14B). Nucleoside **13B** (10 g, 16.3 mmol) was dissolved in anhyd acetonitrile (100 mL) and DBU (2.69 mL, 18.0 mmol) was added. The product slowly precipitated from the reaction mixture. After 2 h the reaction was completed and concentrated in vacuo to facilitate precipitation. The reaction mixture was cooled to -20 °C and the product collected by filtration to afford nucleoside **14B** (7.64 g, 91%) as a white solid material. FAB-MS *m/z* found 517.0 ($[MH]^+$, calcd 517.1); ^{1}H NMR (DMSO-d_6) δ 7.79 (d, J = 1.3, 1H, H6), 7.45-7.32 (m, 5H, Ph), 6.40 (d, J = 6.0, 1H, H1'), 5.60 (dd, J = 6.1, 2.8, 1H, H2'), 4.82 (d, J = 11.5, 1H, C$\underline{H}_2$Ph), 4.70 (d, J = 11.5, 1H, C$\underline{H}_2$Ph), 4.51 (d, J = 2.8, 1H, H3'), 4.43 (d, J = 10.6, 1H), 4.36 (d, J = 6.2, 1H), 4.33 (d, J = 5.9, 1H), 4.25 (d, J = 11.0, 1H) (H5', H1''), 3.22 (s, 3H, Ms), 3.16 (s, 3H, Ms), 1.80 (s, J = 1.1, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 171.5 (C4), 159.1 (C2), 136.9, 132.1, 128.5, 128.1, 127.9 (C6, Ph), 117.1 (C5), 89.1 (C1'), 86.1 (C2'), 85.4 (C4'), 83.7 (C3'), 72.4 (C$\underline{H}_2$Ph), 68.6, 68.0 (C5', C1''), 36.9, 36.8 (Ms), 13.6 (CH_3); Anal. calcd for $C_{20}H_{24}N_2O_{10}S_2$: C, 46.5; H, 4.7; N, 5.4. Found: C, 46.6; H, 4.8; N, 5.3.

1-(3-*O*-Benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl-*β*-D-*threo*-pentofuranosyl)thymine (15B). Nucleoside **14B** (3.70 g, 7.16 mmol) was suspended in a mixture of acetone (160 mL) and aq H_2SO_4 (0.1 M, 160 mL). The mixture was heated to

reflux overnight with stirring. After cooling to rt a white solid precipitated. The volume was reduced to approx. ½ in vacuo and a white solid was isolated by filtration. The solid was washed thoroughly with water and dried in vacuo to give nucleoside **15B** (3.77 g, 98%) as a white solid. FAB-MS *m/z* found 535.0 ($[MH]^+$, calcd 535.1); ^{1}H NMR (DMSO-d_6) δ 11.35 (s, 1H, NH), 7.41-7.32 (m, 6H, H6, Ph), 6.20 (d, J = 5.0, 1H, H1'), 6.10 (d, J = 4.8, 1H, 2'-OH), 4.77 (d, J = 11.9, 1H, C$\underline{H}_2$Ph), 4.67 (d, J = 11.9, 1H, C$\underline{H}_2$Ph), 4.56 (d, J = 10.6, 1H), 4.50-4.41 (m, 3H), 4.32 (d, J = 10.6, 1H), 4.16 (d, J = 3.7, 1H , H3'), 3.25 (s, 3H, Ms), 3.20 (s, 3H, Ms), 1.79 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 163.9 (C4), 150.6 (C2), 137.8, 137.6, 128.4, 127.9, 127.7 (C6, Ph), 108.2 (C5), 84.8 (C1'), 84.3 (C3'), 81.7 (C4'), 73.3 (C2'), 72.3 (C$\underline{H}_2$Ph), 68.1, 67.6 (C5', C1''), 37.0, 36.8 (Ms), 12.2 (CH_3); Anal. calcd for $C_{20}H_{26}N_2O_{11}S_2$: C, 44.9; H, 4.9; N, 5.2. Found: C, 44.5; H, 4.8; N, 5.1.

1-(3-*O*-Benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl-2-*O*-trifluoromethanesulfonyl-*β*-D-*threo*-pentofuranosyl)thymine (16B). Nucleoside **15B** (300 mg, 0.56 mmol) was dissolved in anhyd pyridine (2 × 5 mL) and concentrated in vacuo to remove water traces. The compound was dissolved in a mixture of anhyd dichloromethane (20 mL) and anhyd pyridine (0.45 mL, 5.60 mmol) followed by the addition of DMAP (274 mg, 2.24 mmol). After cooling to 0 °C trifluoromethanesulfonic anhydride (0.19 mL, 1.12 mmol) was added dropwise during 30 min. The reaction mixture was stirred for an additional 1.5 h and poured into ice cooled sat. aq $NaHCO_3$ (20 mL). The organic phase was separated and washed successively with aq HCl (1 M, 2 × 20 mL) and sat. aq $NaHCO_3$ (2 × 20 mL), dried (Na_2SO_4), filtered and evaporated in vacuo. The residue was purified by DCVC (0-100% EtOAc in *n*-heptane v/v) yielding nucleoside **16B** (302 mg, 80%) as a white foam. FAB-MS *m/z* found 667.0 ($[MH]^+$, calcd 667.0); ^{1}H NMR (DMSO-d_6) δ 11.62 (br s, 1H, NH), 7.51 (s, 1H, H6), 7.40-7.33 (m, 5H, Ph), 6.45 (br s, 1H, H1'), 5.91 (t, J = 6.0, 1H, H2'), 4.97 (d, J = 5.7, 1H, H3'), 4.82-4.36 (m, 6H, C$\underline{H}_2$Ph, H5'a, H5'b, H1''a, H1''b), 3.30 (s, 3H, Ms), 3.24 (s, 3H, Ms), 1.81 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 163.3 (C4), 150.0 (C2), 136.5, 128.3, 128.0, 127.8 (C6, Ph), 117.6 (q, J = 320, CF_3), 110.1 (C5), 88.0 (C1'), 81.7, 81.0 (C3', C4'), 73.1

(CH_2Ph), 68.0, 67.6 (C5', C1''), 36.7, 36.6 (Ms), 11.8 (CH_3); Anal. calcd for $C_{21}H_{25}F_3N_2O_{13}S_3$: C, 37.8; H, 3.8; N, 4.2. Found: C, 38.1; H, 3.8; N, 4.1.

1-(2-Azido-3-*O*-benzyl-2-deoxy-5-*O*-methanesulfonyl-4-*C*-(methanesulfonyloxymethyl)-*β*-D-*erythro*-pentofuranosyl)thymine (19).

Method A: To a solution of nucleoside **16B** (215 mg, 0.32 mmol) in anhyd DMF (10 mL) NaN_3 (23 mg, 0.35 mmol) and 15-crown-5 (64 μL, 0.32 mmol) was added. The mixture was stirred at 80 °C for 1 h and then cooled to rt whereupon water (20 mL) was added. The solution was extracted with EtOAc (50 mL) and the organic phase was washed with sat. aq $NaHCO_3$ (2 × 20 mL), dried (Na_2SO_4), filtered and evaporated to dryness in vacuo. The residue was purified by DCVC (50-100% EtOAc in *n*-heptane v/v) yielding nucleoside **19** (164 mg, 91% from **16B**) as a white foam. Analytical data were identical to those reported above.

Method B: A solution of nucleoside **15B** (5.35 g, 10 mmol) in anhyd dichloromethane (300 mL) was cooled to 0 °C. Anhyd pyridine (8.08 mL, 100 mmol) and DMAP (4.89 g, 40 mmol) was added followed by the dropwise addition of triflouromethansulfonic anhydride (3.3 mL, 20 mmol). After 2 h at 0 °C the reaction was quenched by the addition of ice cold sat. aq $NaHCO_3$ (200 mL) and the reaction mixture was transferred to a separatory funnel. The phases were separated and the aq phase was extracted with dichloromethane (200 mL). The combined organic phases were washed with aq HCl (1.0 M, 2 x 300 mL) and sat. aq $NaHCO_3$ (300 mL), dried (Na_2SO_4), filtered and concentrated in vacuo to give a white solid. The solid was dissolved in anhyd DMF (300 mL) and NaN_3 (1.86 g, 30 mmol) was added. After stirring at rt for 4 h brine (300 mL) was added and the mixture was transferred to a separatory funnel. The aq phase was extracted with dichloromethane (3 x 200 mL) and the combined organic phases were dried (Na_2SO_4), filtered and concentrated in vacuo yielding a yellow residue that was purified by DCVC (Ø 5 cm, 25-100% EtOAc in *n*-heptane v/v, 5% increments, 100 mL fractions) affording nucleoside **19** (5.1 g, 91% from **15B**) as a white solid. Analytical data were identical to those reported above.

(1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methansulfonyloxymethyl-3-(thymin-1-yl)-2-oxa-5-thiabicyclo[2:2:1]heptane (23). Nucleoside **16B** (0.10 g, 0.17 mmol) was dissolved in anhyd DMF (1 mL) and potassium thioacetate (25 mg, 0.22 mmol) was added. The reaction was stirred at ambient temperature for 5 h and transferred to a separatory funnel with brine (10 mL). The aq phase was extracted with dichloromethane (3 x 10 mL) and the combined organic phases dried (Na_2SO_4), filtered and evaporated in vacuo to give a yellow liquid. The crude product was dissolved in THF (2 mL) and $LiOH \cdot H_2O$ (35 mg in 1 mL water, 0.84 mmol) was added. After 20 min the reaction was completed and quenched by the addition of glacial acetic acid (0.5 mL). The THF was removed in vacuo and the residue dissolved in dichloromethane (10 mL) and extracted with sat. aq $NaHCO_3$ (2 x 10 mL). The aq phases were extracted with dichloromethane (10 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a yellow liquid that was purified by DCVC (Ø 1 cm, 0-80% EtOAc in *n*-heptane v/v, 2.5% increments, 10 mL fractions). Fractions containing nucleoside **23** were combined and evaporated in vacuo to afford a white powder (36 mg, 47% from **16B**). R_f = 0.38 (80% EtOAc in *n*-heptane, v/v); ESI-MS *m/z* found 455.0 ($[MH]^+$, calcd 455.1); ^{1}H NMR (DMSO-d_6) δ 11.38 (br s, 1H, NH), 7.50 (d, J = 1.1, 1H, H6), 7.36-7.27 (m, 5H, Ph), 5.77 (s, 1H, H1'), 4.68 (d, J = 11.7, 1H), 4.61 (d, J = 11.7, 1H), 4.60 (d, J = 11.7, 1H), 4.56 (d, J = 11.5, 1H) (H5', C$\underline{H}_2$Ph), 4.20 (d, J = 1.8, 1H, H3'), 4.00 (d, J = 2.0, 1H, H2'), 3.29 (s, 3H, Ms), 3.02 (d, J = 10.6, 1H, H1''a), 2.90 (d, J = 10.4, 1H, H1''b), 1.78 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 163.9 (C4), 150.1 (C2), 137.5, 134.1, 128.3, 127.7 (C6, Ph), 108.3 (C5), 90.5 (C1'), 86.6 (C4'), 76.9 (C3'), 70.9, 66.8 (C5', C$\underline{H}_2$Ph), 49.5 (C2'), 36.8 (Ms), 35.1 (C1''), 12.3 (CH_3); Anal. calcd for $C_{19}H_{22}N_2O_7S_2 \cdot 0.33$EtOAc: C, 50.5; H, 5.1; N, 5.8. Found: C, 50.8; H, 5.1; N, 5.8.

(1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methansulfonyloxymethyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (24). To a solution of **19** (5.83 g, 10.4 mmol) in THF (300 mL) at rt aq NaOH (2.0 M, 104 mL, 208 mmol) and PMe_3 in THF (1.0 M, 20.8 mL, 20.8 mmol) was added with stirring. After 8 h the THF was partly removed under reduced pressure. Brine (200 mL) and EtOAc (300 mL) was added and the phases were separated. The aq phase was extracted with EtOAc (2 x 300 mL) and dichloromethane (2 x 300

mL). The combined organic phases were dried (Na_2SO_4), filtered and concentrated in vacuo to give nucleoside **24** (4.22 g, 93%) as a white solid. R_f = 0.15 (10% MeOH in EtOAc, v/v); ESI-MS *m/z* found 438.0 ($[MH]^+$, calcd 438.1); ^{1}H NMR (DMSO-d_6) δ 11.33 (br s, 1H, NH), 7.46 (s, 1H, H6), 7.36-7.27 (m, 5H, Ph), 5.44 (s, 1H, H1'), 4.67 (d, J = 11.7, 1H), 4.59 (d, J = 11.5, 1H), 4.56 (d, J = 11.9, 1H), 4.52 (d, J = 11.7, 1H) (H5', C$\underline{H}_2$Ph), 3.84 (s, 1H, H3'), 3.65 (s, 1H, H2'), 3.26 (s, 3H, Ms), 3.06 (d, J = 10.1, 1H, H1''a), 2.78 (d, J = 9.9, 1H, H1''b), 1.77 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 163.9 (C4), 150.1 (C2), 137.9, 134.7, 128.2, 127.7, 127.6 (C6, Ph), 108.3 (C5), 88.4 (C1'), 85.6 (C4'), 76.3 (C3'), 70.9, 66.6 (C$\underline{H}_2$Ph, C5'), 59.4 (C2'), 50.1 (C1''), 36.9 (Ms), 12.3 (CH_3); Anal. calcd for $C_{19}H_{23}N_3O_7S$: C, 52.1; H, 5.3; N, 9.6. Found: C, 52.0; H, 5.2; N, 9.2.

(1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methansulfonyloxymethyl-5-methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (25). To a solution of **24** (4.22 g, 9.64 mmol) in formic acid (20 mL) formaldehyde (37% aq solution, 20 mL) was added with stirring and the reaction mixture was heated to 80 °C. After 1 h the reaction was diluted with EtOAc (150 mL) and quenched by carefully pouring it into sat. aq $NaHCO_3$ (100 mL). The phases were separated and the organic phase was washed with sat. aq $NaHCO_3$ (4 x 100 mL). The combined aq phases were extracted with dichloromethane (2 x 200 mL). The combined organic phases were dried (Na_2SO_4), filtered and concentrated under reduced pressure. Purification by DCVC (Ø 6 cm, 0-15% MeOH in EtOAc v/v, 1% increments, 100 mL fractions) afforded nucleoside **25** (3.89 g, 90%) as an off-white solid. R_f = 0.30 (10% MeOH in EtOAc, v/v); ESI-MS *m/z* found 452.1 ($[MH]^+$, calcd 452.1); ^{1}H NMR (DMSO-d_6) δ 11.34 (br s, 1H, NH), 7.43 (s, 1H, H6), 7.34-7.28 (m, 5H, Ph), 5.58 (s, 1H, H1'), 4.67 (m, 4H, H5', C$\underline{H}_2$Ph), 3.88 (s, 1H, H3'), 3.58 (s, 1H, H2'), 3.27 (s, 3H, Ms), 2.98 (d, J = 9.7, 1H, H1''a), 2.76 (d, J = 9.7, 1H, H1''b), 2.57 (s, 3H, NCH_3), 1.76 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 163.9 (C4), 149.9 (C2), 137.6 (Ph), 134.6 (C6), 128.3, 127.7 (Ph), 108.4 (C5), 86.1 (C1'), 85.3 (C4'), 77.3 (C3'), 71.0, 66.3 (C$\underline{H}_2$Ph, C5'), 64.9 (C2'), 58.7 (C1''), 40.8 (NCH_3), 36.9 (Ms), 12.3 (CH_3); Anal. calcd for $C_{20}H_{25}N_3O_7S \cdot 0.25\ H_2O$: C, 52.7; H, 5.6; N, 9.1. Found: C, 52.9; H, 5.6; N, 8.9.

(1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-hydroxymethyl-5-methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (26). Compound **25** (3.00 g, 6.64 mmol) was dissolved in anhyd DMF (30 mL) and sodium benzoate (1.93 g, 13.3 mmol) was added. The reaction mixture was heated to 100 °C for 7 h and then cooled to rt. Sodium methoxide (1.44 g, 26.6 mmol) was added and after 1 h the reaction was diluted with dichloromethane (100 mL) and washed with brine (2 x 100 mL). The combined aq phases were extracted with dichloromethane (2 x 50 mL). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissolved in aq HCl (1 M, 15 mL) and lyophilised yielding an off-white solid. Purification by DCVC (Ø 4 cm, 0-10% MeOH in dichloromethane v/v, 0.5% increments, 50 mL fractions) afforded the hydrochloride salt of nucleoside **26** (2.72 g, 98%) as an off-white solid. R_f = 0.19 (7% MeOH in dichloromethane, v/v); ESI-MS *m/z* found 374.1 ($[MH]^+$, calcd 374.2), 408.1, 410.1 ($[MCl]^-$, calcd 408.1, 410.1); ^{1}H-NMR (DMSO-d_6) δ 11.43 (br s, 1H, NH), 7.63 (s, 1H, H6), 7.45-7.29 (m, 5H, Ph), 5.60 (s, 1H, H1'), 4.80 (t, J = 5.7, 1H, 5'-OH), 4.67-4.50 (m, 2H, C$\underline{H}_2$Ph), 3.87 (s, 1H, H3'), 3.67 (d, J = 6.0, 2H, H5'), 3.38 (s, 1H, H2'), 2.88 (d, J = 9.2, 1H, H1''a), 2.66 (d, J = 9.5, 1H, H1''b), 2.57 (s, 3H, NCH_3), 1.75 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 164.0 (C4), 149.8 (C2), 137.0 (Ph), 134.4 (C6), 128.5, 127.8 (Ph), 108.9 (C5), 88.4 (C1'), 88.0 (C4'), 77.8 (C3'), 71.0, ($\underline{C}H_2$Ph), 66.0, 65.7 (C2', C5'), 61.4 (C1''), 40.1 (NCH_3), 12.6 (CH_3); Anal. calcd for $C_{19}H_{23}N_3O_5 \cdot HCl \cdot H_2O$: C, 53.3; H, 6.1; N, 9.8. Found: C, 53.0; H, 6.3; N, 9.6.

(1*R*,3*R*,4*R*,7*S*)-7-Hydroxy-1-hydroxymethyl-5-methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (27). Compound **26** (2.60 g, 6.64 mmol) was dissolved in glacial acetic acid (50 mL) and the reaction flask was evacuated and filled with argon several times. $Pd(OH)_2$ on charcoal (20% moist, 200 mg) was added and the reaction flask was evacuated and filled with hydrogen gas several times. The reaction was stirred vigorously under an atmosphere of hydrogen gas for 8 h. The catalyst was removed by filtration through a plug of celite. The celite was washed thoroughly with hot methanol (200 mL). The solvents were removed in vacuo. The residue was dissolved in water (10 mL) and lyophilised yielding the acetate salt of nucleoside **27** (2.10 g, 97%) as off-white flakes. R_f = 0.11 (0.5% Et_3N, 10% MeOH, 89.5% EtOAc, v/v/v); ESI-MS *m/z* found

284.1 ([MH]$^+$, calcd 284.1). All analytical data were identical to those previously reported.[7]

(1*R*,3*R*,4*R*,7*S*)-1-(4,4'-Dimethoxytrityloxymethyl)-7-hydroxy-5-methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2:2:1]heptane (28). Compound **27** (2.00 g, 5.83 mmol) was dissolved in anhyd pyridine (2 x 50 mL) and concentrated in vacuo. The nucleoside was dissolved in anhyd pyridine (50 mL) and 4,4'-dimethoxytrityl chloride (2.96 g, 8.74 mmol) was added and the reaction was stirred at rt for 9 h. The reaction was concentrated to ½ volume in vacuo and the residue was diluted with EtOAc (100 mL). The organic phase was washed with sat. aq $NaHCO_3$ (3 x 100 mL) and brine (100 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. Purification by DCVC (Ø 4 cm, 0-10% MeOH in EtOAc + 0.5% TEA v/v, 0.5% increments, 50 mL fractions) afforded nucleoside **28** (3.13 g, 92%) as off-white solid. R_f = 0.38 (0.5% Et_3N, 10% MeOH, 89.5% EtOAc, v/v/v); ESI-MS *m/z* found 586.2 ([MH]$^+$, calcd 586.2). All analytical data were identical to those previously reported. (Singh, S. K.; Kumar, R.; Wengel, J. *J.Org.Chem.* **1998**, *63*, 10035-10039)

(1*R*,3*R*,4*R*,7*S*)-7-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-1-(4,4'-dimethoxytrityloxymethyl)-5-methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (29).

Compound **28** (500 mg, 0.85 mmol) was dissolved in anhyd dichloromethane (4 mL) and 4,5-dicyanoimidazole in MeCN (1.0 M, 0.59 mL, 0.59 mmol) was added at ambient temperature with stirring. 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (0.27 mL, 0.85 mmol) was added dropwise to the reaction mixture. After 2 h the reaction was diluted with dichloromethane (10 mL) and transferred to a separatory funnel and extracted with sat. aq $NaHCO_3$ (2 x 15 mL) and brine (15 mL). The combined aq phases were extracted with dichloromethane (10 mL). The organic phases were pooled and dried (Na_2SO_4). After filtration the organic phase was evaporated in vacuo to give nucleoside **29** as a slightly yellow foam (660 mg, 98% yield). R_f = 0.56 (0.5% Et_3N, 10% MeOH, 89.5% EtOAc, v/v/v); ESI-MS *m/z* found 786.3 ([MH]$^+$, calcd 786.4). ^{19}P NMR ($CDCl_3$)

δ149.8, 149.6. (Singh, S. K.; Kumar, R.; Wengel, J. *J.Org.Chem.* **1998**, *63*, 10035-10039)

Example 2; Oligomer Synthesis

All syntheses are carried out in 1 or 15 µmol scale on a MOSS Expedite instrument platform. The synthesis procedures are essentially carried out as described in the instrument manual.

Preparation of the LNA succinyl hemiester

5'-O-Dmt-3'-hydroxy-LNA monomer (500 mg), succinic anhydride (1.2 eq.) and DMAP (1.2 eq.) were dissolved in DCM (35 mL). The reaction was stirred at room temperature overnight. After extractions with NaH_2PO_4 0.1 M pH 5.5 (2x) and brine (1x), the organic layer was further dried with anhydrous Na_2SO_4 filtered and evaporated. The hemiester derivative was obtained in 95 % yield and was used without any further purification.

Preparation of the LNA-CPG resin (controlled pore glass)

The above prepared hemiester derivative (90 µmol) was dissolved in a minimum amount of DMF, DIEA and pyBOP (90 µmol) were added and mixed together for 1 min. This pre-activated mixture was combined with LCAA-CPG (500 Å, 80-120 mesh size, 300 mg) in a manual synthesizer and stirred. After 1.5 h at room temperature, the support was filtered off and washed with DMF, DCM and MeOH. After drying the loading was determined to be 57 µmol/g (see Tom Brown, Dorcas J.S.Brown. Modern machine-aided methods of oligodeoxyribonucleotide synthesis. In: F.Eckstein, editor. Oligonucleotides and Analogues A Practical Approach. Oxford: IRL Press, 1991: 13-14).

Phosphorothioate cycles

5'-O-Dmt (A(bz), C(bz), G(ibu), and T) linked to CPG were deprotected using a solution of 3 % trichloroacetic acid (v/v) in dichloromethane. The resin is washed with acetonitrile. Coupling of phosphoramidites (A(bz), G(ibu), 5-methyl-C(bz)) or T -β-cyanoethylphosphoramidite) is performed by using a solution of 0.08 M of the 5'-O-Dmt-protected amidite in acetonitrile and activation is done by using DCI (4,5 –

dicyanoimidazole) in acetonitrile (0.25 M). Coupling is carried out in 2 minutes. Thiolation is carried out by using Beaucage reagent (0.05 M in acetonitrile) and is allowed to react for 3 minutes. The support is thoroughly washed with acetonitrile and the subsequent capping is carried out by using the standard solution (CAP A) and (CAP B) to cap unreacted 5′- hydroxyl groups. The capping step is then repeated and acetonitrile washing concludes the cycle.

LNA cycles

5′-O-Dmt (locA(bz), locC(bz), locG(ibu) or locT) linked to CPG is deprotected by using the same procedure as above. Coupling is performed by using 5′-O-Dmt (locA(bz), locC(bz), locG(ibu) or locT)-β-cyanoethylphosphoramidite (0.1 M in acetonitrile) and activation is done by DCI (0.25 M in acetonitrile). Coupling is prolonged to 7 minutes. Capping is done by using the standard solutions (CAP A) and (CAP B) for 30 sec. The phosphite triester is oxidized to the more stable phosphate triester by using a standart solution of I_2 and pyridine in THF for 30 sec. The support is washed with acetonitrile and the capping step is repeated. The cycle is concluded by thorough acetonitrile wash.

Cleavage and Deprotection

The oligomers are cleaved from the support and the β-cyanoethyl protecting group removed by treating the support with 35 % NH_4OH 1 h at room temperature. The support is filtered off and the base protecting groups are removed by raising the temperature to 65 °C for 4 hours. The ammonia is then removed by evaporation.

Purification

The oligoes are either purified by (reversed-phase) RP-HPLC or (anion exchange) AIE.

RP-HPLC:

Column: VYDAC™ cat. No. 218TP1010 (vydac)

Flow rate: 3 mL/min

Buffer: A 0.1 M ammonium acetate pH 7.6

B acetonitrile

Gradient:

Time	0	10	18	22	23	28
B %	0	5	30	100	100	0

IE:

Column: Resource™ 15Q (*amersham pharmacia biotech*)

Flow rate: 1.2 mL/min

Buffer: A 0.1 M NaOH

B 0.1 M NaOH, 2,0 M NaCl

Gradient:

Time	0	1	27	28	32	33
B %	0	25	55	100	100	0

Abbreviations

Dmt: Dimethoxytrityl

DCI: 4,5-Dicyanoimidazole

DMAP: 4-Dimethylaminopyridine

DCM: Dichloromethane

DMF: Dimethylformamide

THF: Tetrahydrofurane

DIEA: *N,N*-diisopropylethylamine

PyBOP: Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate

Bz: Benzoyl

Ibu: Isobutyryl

Beaucage: 3H-1,2-Benzodithiole-3-one-1,1-dioxide

LocA, locC, locG, locT: LNA-monomers (LNA-locked nucleic acid)

Tm measurement

Melting curves were recorded with a Perkin Elmer UV/Vis spectrophotometer lambda 40 attached to a PTP-6 Peltier System. Oligonucleotides were dissolved in salt buffer (10 mM phosphate buffer, 100 mM NaCl, 0,1 mM EDTA, pH 7.0) using the two complementary strands at 1.5 µM and 1 cm path-length cells. Samples were denatured at 95°C for 3 min and slowly cooled to 20°C prior to measurements. Melting curves were

recorded at 260 nm using a heating rate of 1°C/min, a slit of 2 nm and a response of 0.2 s. Tm values were obtained from the maxima of the first derivatives of the melting curves.

Example 3; Test of design of the oligomeric compound

It was of our interest to evaluate the antisense activity of oligonucleotides with different designs, in order to prove the importance of choosing the best design for an oligonucleotide targeting Ha-Ras. For this purpose, we set up an *in vitro* assay that would allow us to screen many different oligonucleotide designs by measuring the activity of the firefly (Photinus pyralis) luciferase after down-regulation by antisense oligonucleotides. Figure 1 contains an illustration of the designs mentioned in the text.

In a first screen, designs containing β-D-oxy-LNA, which were all targeting the same motif within the mRNA were evaluated. Designs consisting of gapmers with a different gap-size, a different load of phosphorothioate internucleoside linkages, and a different load of LNA were tested. Headmers and tailmers with a different load of β-D-oxy-LNA, a different load of phosphorothioate internucleoside linkages and a different load of DNA were prepared. Mixmers of various compositions, which means that bear an alternate number of units of β-D-oxy-LNA, α-L-LNA and DNA, were also analysed in the *in vitro* assay. Moreover, LNA derivatives were also included in different designs, and their antisense activity was assessed. The importance of a good design is reflected by the data that can be obtained in a luciferase assay. The luciferase expression levels are measured in %, and give an indication of the antisense activity of the different designs containing β-D-oxy-LNA and LNA derivatives. We can easily see that some designs are potent antisense oligonucleotides, while others give moderate to low down-regulation levels. Therefore, a close correlation between good antisense activity and optimal design of an oligonucleotide is very evident. We appreciated good levels of down-regulation with various designs. Gapmers with gaps of 7-10 nt DNA and thiolation all over the backbone or with thiolation exclusively in the gap and PO in the flanks showed good results. These designs contain β-D-oxy-LNA or LNA derivatives. Headmers of 6 nt and 8 nt β-D-oxy-LNA also presented good levels of down-regulation, when the phosphorothioate internucleoside linkages are all over the backbone or only in the DNA-segment. Different mixmers gave good antisense activity in the luciferase assay. The alternate number of

units of each α-L-oxy-LNA, β-D-oxy-LNA or DNA composition defines the mixmers, see figure 1. A mixmer **3-9-3-1**, which has a deoxynucleoside residue at the 3'-end showed significant levels of down-regulation. In a mixmer **4-1-1-5-1-1-3**, we placed two α-L-oxy-LNA residues interrupting the gap, being the flanks β-D-oxy-LNA. Furthermore, we interrupted the gap with two α-L-oxy-LNA residues, and substituted both flanks with α-L-oxy-LNA. Both designs presented significant levels of down-regulation. The presence of α-L-oxy-LNA might introduce a flexible transition between the North-locked flanks (oxy-LNA) and the α-L-oxy-LNA residue by spiking in deoxynucleotide residues. It is also interesting to study design **4-3-1-3-5** where a α-L-oxy-LNA residue interrupts the DNA stretch. In addition to the α-L-oxy-LNA in the gap, we also substituted two oxy-LNA residues at the edges of the flanks with two α-L-oxy-LNA residues. The presence of just one β-D-oxy-LNA residue (design **4-3-1-3-5**) interrupting the stretch of DNAs in the gap results in a dramatic loss of down-regulation. Just by using α-L-oxy-LNA instead, the design shows significant down-regulation at 50nM oligonucleotide concentration. The placement of α-L-oxy-LNA in the junctions and one α-L-oxy-LNA in the middle of the gap also showed down-regulation.

α-L-oxy-LNA reveals to be a potent tool enabling the construction of different mixmers, which are able to present high levels of antisense activity. Other mixmers such as **4-1-5-1-5** and **3-3-3-3-3-1** can also be prepared. We can easily see that some designs are potent antisense oligonucleotides, while others give moderate to low down-regulation levels. Therefore, again a close correlation between good antisense activity and optimal design of an oligonucleotide is very evident.

Assay

X1/5 Hela cell line (ECACC Ref. No: 95051229), which was stably transfected with a "tet-off" luciferase system, was used. In the absence of tetracycline the luciferase gene is expressed constitutively. The expression can be measured as light in a luminometer, when the luciferase substrate, luciferin is added. The X1/5 Hela cell line was grown in Minimun Essential Medium Eagle (Sigma M2279) supplemented with 1x Non Essential Amino Acid (Sigma M7145), 1x Glutamax I (Invitrogen 35050-038), 10 % FBS calf serum, 25 µg/ml Gentamicin (Sigma G1397), 500 µg/ml G418 (Invitrogen 10131-027)

and 300 µg/ml Hygromycin B (Invitrogen 10687-010). The X1/5 Hela cells were seeded at a density of 8000 cells per well in a white 96 well plate (Nunc 136101) the day before the transfection. Before the transfection, the cells were washed one time with OptiMEM (Invitrogen) followed by addition of 40 µl OptiMEM with 2µg/ml of Lipofectamine2000 (Invitrogen). The cells were incubated for 7 minutes before addition of the oligonucleotides. 10 µl of oligonucleotide solutions were added and the cells were incubated for 4 h at 37°C and 5 % CO_2. After the 4 h incubation, the cells were washed once in OptiMEM and growth medium was added (100 µl). The luciferase expression was measure the next day. Luciferase expression was measured with the Steady-Glo luciferase assay system from Promega. 100 µl of the Steady-Glo reagent was added to each well and the plate was shaken for 30 s at 700 rpm. The plate was read in Luminoskan Ascent instrument from ThermoLabsystems after 8minof incubation to complete total lysis of the cells. The luciferase expression is measured as Relative Light Units per seconds (RLU/s). The data was processed in the Ascent software (v2.6) and graphs were drawn in SigmaPlot2001.

Example 4; In vitro model: Cell culture

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. Target can be expressed endogenously or by transient or stable transfection of a nucleic acid encoding said nucleic acid. The expression level of target nucleic acid can be routinely determined using, for example, Northern blot analysis, Real-Time PCR, Ribonuclease protection assays. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen.

Cells were cultured in the appropriate medium as described below and maintained at 37°C at 95-98% humidity and 5% CO_2. Cells were routinely passaged 2-3 times weekly.

15PC3: The human prostate cancer cell line 15PC3 was kindly donated by Dr. F. Baas, Neurozintuigen Laboratory, AMC, The Netherlands and was cultured in DMEM (Sigma) + 10% fetal bovine serum (FBS) + Glutamax I + gentamicin

A549: The human non-small cell lung cancer cell line A549 was purchased from ATCC, Manassas and was cultured in DMEM (Sigma) + 10% FBS + Glutamax I + gentamicin

MCF7: The human breast cancer cell line MCF7 was purchased from ATCC and was cultured in Eagle MEM (Sigma) + 10% FBS + Glutamax I + gentamicin

SW480: The human colon cancer cell line SW480 was purchased from ATCC and was cultured in L-15 Leibovitz (Sigma) + 10% FBS + Glutamax I + gentamicin

SW620: The human colon cancer cell line SW620 was purchased from ATCC and was cultured in L-15 Leibovitz (Sigma) + 10% FBS + Glutamax I + gentamicin

HT29: The human prostate cancer cell line HT29 was purchased from ATCC and was cultured in McCoy's 5a MM (Sigma) + 10% FBS + Glutamax I + gentamicin

NCI H23: The human non-small-cell lung cancer cell line was purchased from ATCC and was cultured in RPMI 1640 with Glutamax I (Gibco) + 10% FBS + HEPES + gentamicin

HCT-116: The human colon cancer cell line HCT-116 was purchased from ATCC and was cultured in McCoy's 5a MM + 10% FBS + Glutamax I + gentamicin

MDA-MB-231: The human breast cancer cell line MDA-MB-231 was purchased from ATCC and was cultured in L-15 Leibovitz + 10% FBS + Glutamax I + gentamicin

MDA-MB-435s: The human breast cancer cell line MDA-MB-435s was purchased from ATCC and was cultured in L-15 Leibovitz + 10% FBS + Glutamax I + gentamicin

DMS273: The human small-cell lung cancer cell line DMS273 was purchased from ATCC and was cultured in Waymouth with glutamine (Gibco) + 10% FBS + gentamicin

PC3: The human prostate cancer cell line PC3 was purchased from ATCC and was cultured in F12 Coon's with glutamine (Gibco) + 10% FBS + gentamicin

Example 5; In vitro model: Treatment with antisense oligonucleotide

The cells were treated with oligonucleotide using the cationic liposome formulation LipofectAMINE 2000 (Gibco) as transfection vehicle. Cells were seeded in 12-well cell culture plates (NUNC) and treated when 80-90% confluent. Oligo concentrations used

ranged from 125 nM to 0,2 nM final concentration. Formulation of oligo-lipid complexes were carried out essentially as described in Dean et al. (Journal of Biological Chemistry 1994, 269, 16416-16424) using serum-free OptiMEM (Gibco) and a final lipid concentration of 10 µg/mlLipofectAMINE 2000 in 500 µl total volume. Cells were incubated at 37°C for 4 hours and treatment was stopped by removal of oligo-containing culture medium. Cells were washed and serum-containing media was added. After oligo treatment cells were allowed to recover for 18 hours before they were harvested for RNA or protein analysis.

Example 6; In vitro model: Extraction of RNA and cDNA synthesis

Total RNA Isolation

Total RNA was isolated either using RNeasy mini kit (Qiagen cat. no. 74104) or using the Trizol reagent (Life technologies cat. no. 15596). For RNA isolation from cell lines, RNeasy is the preferred method and for tissue samples Trizol is the preferred method.
Total RNA was isolated from cell lines using the Qiagen RNA OPF Robot – BIO Robot 3000 according to the protocol provided by the manufacturer. Tissue samples were homogenised using an Ultra Turrax T8 homogeniser (IKA Analysen technik) and total RNA was isolated using the Trizol reagent protocol provided by the manufacturer.

First strand synthesis

First strand synthesis was performed using OmniScript Reverse Transcriptase kit (cat# 205113, Qiagen) according to the manufacturers instructions.
For each sample 0.5 µg total RNA was adjusted to 12 µl each with RNase free H_2O and mixed with 2 µl poly $(dT)_{12-18}$ (2.5 µg/ml) (Life Technologies, GibcoBRL, Roskilde, DK), 2 µl dNTP mix (5 mM each dNTP), 2 µl 10x Buffer RT, 1 µl RNAguard™Rnase INHIBITOR (33.3U/ml), (cat# 27-0816-01, Amersham Pharmacia Biotech, Hørsholm, DK) and 1 µl OmniScript Reverse Transcriptase (4 U/µl) followed by incubation at 37°C for 60 minutes and heat inactivation of the enzyme at 93°C for 5 minutes.

Example 7; In vitro model: Analysis of Oligonucleotide Inhibition of Ha-ras Expression by Real-time PCR

Antisense modulation of Ha-ras expression can be assayed in a variety of ways known in the art. For example, Ha-ras mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR. Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or mRNA.

Methods of RNA isolation and RNA analysis such as Northern blot analysis is routine in the art and is taught in, for example, Current Protocols in Molecular Biology, John Wiley and Sons.

Real-time quantitative (PCR) can be conveniently accomplished using the commercially available iQ Multi-Color Real Time PCR Detection System, available from BioRAD.

Real-time Quantitative PCR Analysis of Ha-ras mRNA Levels

Quantitation of mRNA levels was determined by real-time quantitative PCR using the iQ Multi-Color Real Time PCR Detection System (BioRAD) according to the manufacturers instructions.

Real-time Quantitative PCR is a technique well known in the art and is taught in for example Heid et al. Real time quantitative PCR, Genome Research (1996), 6: 986-994.

Platinum Quantitative PCR SuperMix UDG 2x PCR master mix was obtained from Invitrogen cat# 11730. Primers and TaqMan® probes were obtained from MWG-Biotech AG, Ebersberg, Germany

Probes and primers to human Ha-ras were designed to hybridise to a human Ha-ras sequence, using published sequence information (GenBank accession number J00277, incorporated herein as SEQ ID NO:1).

For human Ha-ras the PCR primers were:

forward primer: 5' gccggatgcaggaaggag 3' (final concentration in the assay; 0.3 μM)(SEQ ID NO: 80)

reverse primer: 5' gctccagcagccctlcctt 3' (final concentration in the assay; 0.3 μM)(SEQ ID NO: 81) and the PCR probe was: 5' FAM- cgtccttccttcctcctccttccgtctg -TAMRA 3' (final concentration in the assay; 0.1 μM)(SEQ ID NO: 82)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA quantity was used as an endogenous control for normalizing any variance in sample preparation.

The sample content of human GAPDH mRNA was quantified using the human GAPDH ABI Prism Pre-Developed TaqMan Assay Reagent (Applied Biosystems cat. no. 4310884E) according to the manufacturers instructions.

For quantification of mouse GAPDH mRNA the following primers and probes were designed: Sense primer 5'aaggctgtgggcaaggtcatc 3' (0.3 μM final concentration),
antisense primer 5' gtcagatccacgacggacacatt (0.6 μM final concentration),
TaqMan probe 5` FAM-gaagctcactggcatggcatggccttccgtgttc-TAMRA 3' (0.2 μM final concentration).

Real time PCR

The cDNA from the first strand synthesis performed as described in example 8 was diluted 2-20 times, and analyzed by real time quantitative PCR. The primers and probe were mixed with 2 x Platinum Quantitative PCR SuperMix UDG (cat. # 11730, Invitrogen) and added to 3.3 μl cDNA to a final volume of 25 μl. Each sample was analysed in triplicates. Assaying 2 fold dilutions of a cDNA that had been prepared on material purified from a cell line expressing the RNA of interest generated standard curves for the assays. Sterile H_2O was used instead of cDNA for the no template control. PCR program: 50° C for 2 minutes, 95° C for 10 minutes followed by 40 cycles of 95° C, 15 seconds, 60° C, 1 minutes.
Relative quantities of target mRNA sequence were determined from the calculated Threshold cycle using the iCycler iQ Real-time Detection System software.

Example 8; in vitro analysis: Northern Blot Analysis of Ha-ras mRNA Levels

Northern blot analysis was carried out by procedures well known in the art essentially as described in Current Protocols in Molecular Biology, John Wiley & Sons.

The hybridisation probe was obtained by PCR-amplification of a 381 bp fragment from 15PC3 cDNA obtained by reverse transcription PCR as described in example 8. The reaction was carried out using primers 5' aatctcggcaggctcaggac 3' (forward) and 5' gggatgttcaagacagtctgtgc 3' (reverse) at 0,5 µM final concentration each, 200 nM each dNTP, 1,5 mM $MgCl_2$ and Platinum Taq DNA polymerase (Invitrogen cat. no. 10966-018).
The DNA was amplified for 40 cycles on a Perkin Elmer 9700 thermocycler using the following program: 94°C for 2 min. then 40 cycles of 94°C for 30 sec. and 72°C for 30 sec. with a decrease of 0.5°C per cycle followed by 72°C for 7 min.

The amplified PCR product was purified using S-400 MicroSpin columns (Amersham Pharmacia Biotech cat. no. 27-5140-01) according to the manufacturers instructions and quantified by spectrophotometry.

The hybridisation probe was labelled using Redivue™ [α-^{32}P]dCTP 3000 Ci/mmol (Amersham Pharmacia Biotech cat. no. AA 0005) and Prime-It RmT labeling kit (Stratagene cat. no. 300392) according to the manufacturers instructions and the radioactively labeled probe was purified using S-300 MicroSpin columns (Amersham Pharmacia Biotech cat. no. 27-5130-01).
Before use, the probe was denatured at 96°C and immediately put on ice.

Samples of 1-5 µg of total RNA purified as described in example 7 were denatured and size separated on a 2,2 M formaldehyde/MOPS agarose gel.
RNA was transferred to positively charged nylon membrane by downward capillary transfer using the TurboBlotter (Schleicher & Schuell) and the RNA was immobilised to the membrane by UV crosslinking using a Stratagene crosslinker.

The membrane was prehybridised in ExpressHyb Hybridization Solution (Clontech cat. No. 8015-1) at 60°C and the probe was subsequently added for hybridisation. Hybridisation was carried out at 60°C and the blot was washed with low stringency wash buffer (2 x SSC, 0,1% SDS) at room temperature and with high stringency wash buffer (0,1 x SSC, 0,1% SDS) at 50°C.
The blot was exposed to Kodak storage phosphor screens and scanned in a BioRAD FX molecular imager. Ha-ras mRNA levels were quantified by Quantity One software (BioRAD)

Equality of RNA sample loading was assessed by stripping the blot in 0,5% SDS in H_2O at 85°C and reprobing with a labelled GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe obtained essentially as described above using the primers 5' aac gga ttt ggt cgt att 3' (forward) and 5' taa gca gtt ggt ggt gca 3' (reverse).

Example 9: In vitro analysis: Western blot analysis of Ha-ras protein levels
Protein levels of Ha-ras can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA, RIA (Radio Immuno Assay) or fluorescence-activated cell sorting (FACS). Antibodies directed to Ha-ras can be identified and obtained from a variety of sources, such as Upstate Biotechnologies (Lake Placid, USA), Novus Biologicals (Littleton, Colorado), Santa Cruz Biotechnology (Santa Cruz, California) or can be prepared via conventional antibody generation methods.

Western blotting:
To measure the effect of treatment with antisense oligonucleotides against Ha-ras, protein levels of Ha-ras in treated and untreated cells were determined using western blotting.
After treatment with oligonucleotide as described in example 5, cells were harvested in ice-cold lysis buffer (50 mM Tris, pH 6,8, 10 mM NaF, 10% glycerol, 2,5% SDS, 0,1 mM natrium-orthovanadate, 10 mM β-glycerol phosphate, 10 mM dithiothreitol (DTT), Complete protein inhibitor cocktail (Boehringer Mannheim)). The lysate was stored at -80°C until further use.

Protein concentration of the protein lysate was determined using the BCA Protein Assay Kit (Pierce) as described by the manufacturer.

SDS gel electrophoresis:

Protein samples prepared as described above were thawed on ice and denatured at 96°C for 3 min.

Samples were loaded on 1,0 mm 4-20% NuPage Tris-glycine gel (Invitrogen) and gels were run in TGS running buffer (BioRAD) in an Xcell II Mini-cell electrophoresis module (Invitrogen).

Semi-dry blotting:

After electrophoresis, the separated proteins were transferred to a polyvinyliden difluoride (PVDF) membrane by semi-dry blotting.

The blotting procedure was carried out in a Semi-Dry transfer cell (CBS Scientific Co.) according to the manufacturers instructions. The membrane was stained with amidoblack to visualise transferred protein and was stored at 4°C until further use.

Immunodetection:

To detect the desired protein, the membrane was incubated with either polyclonal or monoclonal antibodies against the protein.

The membrane was blocked in blocking buffer (5% skim milk powder dissolved in PBST-buffer (PBS + 0,1% Tween-20)), washed briefly in PBS-buffer and incubated with primary antibody in blocking buffer at room temperature.

The following primary and secondary antibodies and concentrations/dilutions were used:

Polyclonal rabbit anti-human H-ras antibody (cat. # sc-520, Santa Cruz) 1:200

Monoclonal mouse anti-human tubulin Ab-4 (cat.# MS-719-P1, NeoMarkers) 1:500

Peroxidase-conjugated Swine Anti-Rabbit Immunoglobulins (code no. P0399, DAKO) 1:3000

Peroxidase-conjugated Goat Anti-Mouse Immunoglobulins (code no. P0447, DAKO) 1:1000

After incubation with the primary antibody the membrane was washed briefly in PBS followed by 3 additional 10 minutes washes in PBST with agitation at room temperature and incubated with a peroxidase conjugated secondary antibody in blocking buffer at room temperature. The membrane was then washed in PBS followed by 3 additional 10 minutes washes in PBST with agitation at room temperature. After the last wash the membrane was incubated with ECL$^+$ Plus reagent (Amersham) and chemiluminescens was detected using VersaDoc chemiluminescens detection system (BioRAD) or X-omat film (Kodak). The membrane was stripped in ddH_2O by incubation for 1 minute at 96° C. After stripping, the membrane was put in PBS and stored at 4° C.

Example 10; In vitro analysis: Antisense Inhibition of Human Ha-ras Expression by antisense oligonucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Ha-ras RNA, using published sequences (GenBank accession number J00277, incorporated herein as SEQ ID NO: 1, Figure 7). The oligomeric compounds with 16 nucleotides in length are shown in Table 1 having a CUR NO and a SEQ ID NO. "Target site" indicates the first nucleotide number on the particular target sequence to which the oligonucleotide binds. Table 2 shows low IC50 of four compounds.

Table 1 Oligomeric compounds of the invention

Oligomeric compounds were evaluated for their potential to knockdown Ha-ras in 15PC3. Transcript steady state was monitored by Real-time PCR and normalised to the GAPDH transcript steady state.

SEQ ID NO & Cureon No	% Inhibition at 25 nM oligo concen-tration	Target site	Oligomeric compound Sequence 5'-3'	Specific design of Oligomeric compound Capital letters β-D-oxy-LNA
2 CUR2709	29	1742 (260 K-ras)	ATTCGTCCACAAAATG	$A_sT_sT_sC_sg_st_sc_sc_sa_sc_sa_sa_sA_sA_sT_sG$
3 CUR2710	60	1733 (323 N-ras)	CAAAATGGTTCTGGAT	$C_sA_sA_sA_sa_st_sg_sg_st_st_sc_st_sG_sG_sA_sT$
4 CUR2711	67	1745 (263 K-ras)	CGTATTCGTCCACAAA	$C_sG_sT_sA_st_st_sc_sg_st_sc_sc_sa_sC_sA_sA_sA$
5 CUR2712	62	2158	CACACACAGGAAGCCC	$C_sA_sC_sA_sc_sa_sc_sa_sg_sg_sa_sa_sG_sC_sC_sC$

6 CUR2713	90	3701	CCCATCTGTGCCCGAC	$C_sC_sC_sA_st_sc_st_sg_st_sg_sc_sc_sC_sG_sA_sC$
7 CUR2714	63	2168 (491 N-ras)	TGATGGCAAACACACA	$T_sG_sA_sT_sg_sg_sc_sa_sa_sa_sc_sa_sC_sA_sC_sA$
8 CUR2715	57	2182	AGACTTGGTGTTGTTG	$A_sG_sA_sC_st_st_sg_sg_st_sg_st_st_sG_sT_sT_sG$
9 CUR2716	67	2383	GTCCTTCACCCGTTTG	$G_sT_sC_sC_st_st_sc_sa_sc_sc_sc_sg_sT_sT_sT_sG$
10 CUR2717	66	2393	CGTCATCCGAGTCCTT	$C_sG_sT_sC_sa_st_sc_sc_sg_sa_sg_st_sC_sC_sT_sT$
11 CUR2718	49	2431	AGCCAGGTCACACTTG	$A_sG_sC_sC_sa_sg_sg_st_sc_sa_sc_sa_sC_sT_sT_sG$
12 CUR2719	77	2453	GCCGAGATTCCACAGT	$G_sC_sC_sG_sa_sg_sa_st_st_sc_sc_sa_sC_sA_sG_sT$
13 CUR2720	68	3228 (629 K-ras)	CATCCTCCACTCCCTG	$C_sA_sT_sC_sc_st_sc_sc_sa_sc_st_sc_sC_sC_sT_sG$
14 CUR2721	89	3253	ATCTCACGCACCAACG	$A_sT_sC_sT_sc_sa_sc_sg_sc_sa_sc_sc_sA_sA_sC_sG$
15 CUR2722	99	3506	TCCTCCTTCCGTCTGC	$T_sC_sC_sT_sc_sc_st_st_sc_sc_sg_st_sC_sT_sG_sC$
16		1610	GGTCTCCTGCCCCACC	
17		1626	CGGGGTCCTCCTACAG	
18		1642	TCAGGGGCCTGCGGCC	
19		1658	ATTCCGTCATCGCTCC	
20		1674	ACCACCACCAGCTTAT	
21		1690	CACACCGCCGGCGCCC	
22		1706	TCAGCGCACTCTTGCC	
23		1738	GTCCACAAAATGGTTC	
24		1754	TAGTGGGGTCGTATTC	
25		2037	CGGTAGGAATCCTCTA	
26		2053	AATGACCACCTGCTTC	
27		2069	GGCACGTCTCCCCATC	
28		2085	TCCAGGATGTCCAACA	
29		2101	CTCCTGGCCGGCGGTA	
30		2117	GCATGGCGCTGTACTC	
31		2133	CGCATGTACTGGTCCC	
32		2149	GAAGCCCTCCCCGGTG	
33		2165	TGGCAAACACACACAG	
34		2181	GACTTGGTGTTGTTGA	
35		2197	GTGGATGTCCTCAAAA	
36		2213 Exon-exon junction	TCTGCTCCCTGTACTG	
37		2382	TCCTTCACCCGTTTGA	
38		2398	GGGCACGTCATCCGAG	
39		2414	TCCCCACCAGCACCAT	
40		2430	GCCAGGTCACACTTGT	
41		2446	TTCCACAGTGCGTGCA	
42		2462	CCTGAGCCTGCCGAGA	
43		2478	TAGCTTCGGGCGAGGT	

44		2494	GATGTAGGGGATGCCG
45		2510	TCTTGGCCGAGGTCTC
46		2526 Exon-exon junction	TCCACTCCCTGCCGGG
47		3239	CGTGTAGAAGGCATCC
48		3255	GGATCTCACGCACCAA
49		3271	CGCAGCTTGTGCTGCC
50		3287	AGGAGGGTTCAGCTTC
51		3303	CGGGGCCACTCTCATC
52		3319	TTGCAGCTCATGCAGC
53		3335	TCAGGAGAGCACACAC
54		3459	CTGAGCTTGTGCTGCG
55		3475	CCGGCACCTCCATGTC
56		3491	CACCTCCTTCCTGCAT
57		3507	CTCCTCCTTCCGTCTG
58		3523	CTTCCGTCCTTCCTTC
59		3539	CTTCCTTCCTTCCTTG
60		3555	CTGGGCTCCAGCAGCC
61		3571	CACGGTCCCGGGGTGA
62		3587	TGCAGTCACCTCGGCC
63		3603	CCTCCCTGGGAGGGTC
64		3619	GACAGTCTGTGCACAG
65		3635	CATTTGGGATGTTCAA
66		3651	GCTGGGGTTCCGGTGG
67		3667	GGGAGGGGAGCTAAGG
68		3683	GGGCCCACAGAGGCCT
69		3699	CATCTGTGCCCGACAA
70		3715	TAATTTACTGTGATCC
71		3731	TTTCAAGACCATCCAA
72		1722	TGGATCAGCTGGATGG
73		1690	CACACCGTCGGCGCCC
74		2101	CTCCAGGCCGGCGGTA

Table 2 IC50 of the LNA (β-D-oxy-LNA) containing oligomeric compounds

SEQ ID NO	Cureon No	IC50 in 15PC3
3	CUR2710	0.2
5	CUR2713	0.08
14	CUR2721	0.6
15	CUR2722	0.1

Example 11; *In vivo* model: Tumour growth inhibition of human tumour cells grown *in vivo* by systemic treatment with antisense oligonucleotides

Female NMRI athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize for at least one week before entering experiments. Human

cancer cells typically 10^6 cells suspended in 300 µl matrigel (BD Bioscience), were subcutaneously injected into the flanks of 7-8 week old NMRI athymic female nude mice. When the tumour growth was established, typically 7-12 days post tumour cell injection; different antisense oligonucleotides were administrated at 5 mg/kg/day for up to 28 days using ALZET osmotic pumps implanted subcutaneously. Prior to dorsal implantation the pumps were incubated overnight at room temperature in sterile PBS to start the pumps. Control animals received saline alone for the same period. Each experimental group included at least 5 mice. Anti-tumour activities were estimated by the inhibition of tumour volume. Tumour growth was followed regularly by measuring 2 perpendicular diameters. Tumour volumes were calculated according to the formula $(\pi \times L \times D^2/6)$, where L represents the largest diameter and D the tumour diameter perpendicular to L. At the end of treatment the animals were sacrificed and tumour weights were measured. Mean tumour volume and weights of groups were compared using Mann-Whitney's test. All analysis was made in SPSS version 11.0 for windows.

Example 12; *In vivo* analysis: Inhibition of Ha-ras protein level in human tumour cells grown *in vivo* systemic treatment with antisense oligonucleotides

Female NMRI athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize for at least one week before entering experiments. Human cancer cells typically 10^6 cells suspended in 300 µl matrigel (BD Bioscience), were subcutaneously injected into the flanks of 7-8 week old NMRI athymic female nude mice. When the tumour growth was established, typically 7-12 days post tumour cell injection; different antisense oligonucleotides were administrated at 5 mg/kg/day for up to 28 days using ALZET osmotic pumps implanted subcutaneously. Prior to dorsal implantation the pumps were incubated overnight at room temperature in sterile PBS to start the pumps. Control animals received saline alone for the same period. Each experimental group included at least 5 mice. To measure if the antisense oligonucleotides have an inhibitory effect on protein levels, Western blot analysis was performed.
At the end of treatment period mice were anaesthetised and the tumours were excised and immediately frozen in liquid nitrogen.

The tumours were homogenized in lysis buffer (i.e. 20 m*M* Tris-Cl [pH 7.5]; 2% Triton X-100; 1/100 vol. Protease Inhibitor Cocktail Set III (Calbiochem); 1/100 vol. Protease Inhibitor Cocktail Set II (Calbiochem)) at 4°C with the use of a motor-driven homogeniser. 500 µl lysis buffer was applied per 100 mg tumour tissue. Tumour lysates from each group of mice were pooled and centrifuged at 13.000 *g* for 5 min at 4°C to remove tissue debris. Protein concentrations of the tumour extracts were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford).

The protein extracts (50-100 µg) were fractionated on a gradient SDS-PAGE gel spanning from 4-20% and transferred to PVDF membranes and visualized by aminoblack staining. The expression of Ha-ras was detected with anti-human Ha-ras antibody followed by horseradish peroxidase-conjugated anti-goat IgG (DAKO). Immunoreactivity was detected by the ECL Plus (Amersham biotech) and quantitated by a Versadoc 5000 lite system (Bio-Rad).

Example 13; Biodistribution of oligonucleotides in mice

Female NMRI athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize for at least one week before entering experiments. Human cancer cells typically 10^6 cells suspended in 300 µl matrigel (BD Bioscience) were subcutaneously injected into the flanks of 7-8 week old NMRI athymic female nude mice. When tumour growth was evident, tritium labelled oligonucleotides were administrated at 5 mg/kg/day for 14 days using ALZET osmotic pumps implanted subcutaneously. The oligonucleotides were tritium labeled as described by Graham MJ et al. (J Pharmacol Exp Ther 1998; 286(1): 447-458). Oligonucleotides were quantitated by scintillation counting of tissue extracts from all major organs (liver, kidney, spleen, heart, stomach, lungs, small intestine, large intestine, lymph nodes, skin, muscle, fat, bone, bone marrow) and subcutaneous transplanted human tumour tissue.

Example 14; *In vivo* model: Inhibition of lung metastases from human xenotransplanted breast cancer in nude mice by systemic treatment with antisense

oligonucleotides

Female BalbC-a athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize at for least one week before entering experiments. Human breast cancer (HABC1) fragments from donor mice of approximately 1 mm^3 in size were subcutaneously implanted in the flanks of athymic female BalbC-a mice. The HABC1 breast cancer line was obtained from Anne-Marie Engel, Bartholin Institute, Denmark. When tumour growth was established (e.i. the product of perpendicular diameters were 25-36 mm^2) oligonucleotides were administrated at 5 mg/kg/day for 28 days using ALZET osmotic pumps implanted subcutaneously. Prior to dorsal implantation the pumps were incubated overnight at room temperature in sterile PBS to start the pumps. Control animals received saline alone for the same period. Each experimental group included at least 10 mice.

Anti-tumour activities were estimated by the inhibition of volume of the subcutaneously implanted tumour and by quantitation of micro-metastases in the lung tissue. Tumour growth was followed regularly by measuring 2 perpendicular diameters. Tumour volumes were calculated according to the formula ($\pi \times L \times D^2/6$), where L represents the largest diameter and D the tumour diameter perpendicular to L. At the end of treatment mice were sacrificed and both lungs were excised and embedded in paraffin. Lung metastases from the subcutaneously implanted human breast cancer cells were quantitated on H&E stained tissue sections. The number of micro-metastases in the lungs of different treatment groups was statistically compared using Mann-Whitney test.

Example 15; In vitro superiority

Human prostate cancer cell line 15PC3 was maintained as described in example 4.

Cells were transfected using the lipid transfection reagent DAC-30 (Eurogentec) as described in Ten Asbroek et al.(2000), Polymorphisms in the large subunit of human RNA polymerase II as target for allele-specific inhibition. Nucleic Acid Research 28: 1133-1138. Oligo concentrations used for transfection were 200 nM, 400 nM and 800 nM final concentration. Expression levels of Ha-ras RNA was determined by Northern blot analysis using a protocol as described in Ten Asbroek et al.(2000), Polymorphisms in the

large subunit of human RNA polymerase II as target for allele-specific inhibition (see Figure 2). Nucleic Acid Research 28: 1133-1138. Hybridisation probes were generated by RT-PCR and subsequent cloning into pGEM-T Easy vector (Promega). The Ha-ras probe consisted of the sequence from position 1657-3485 (exon sequences only) of Seq ID NO. 1 (Figure 7).

Example 16, In vivo superiority and specificity of LNA oligomeric compounds compared to corresponding Phosphorothioates

Table 3 shows the antisense compound prepared for the In vivo superiority and specificity analysis.

Table 3 Oligonucleotides prepared for the In vivo superiority and specificity analysis

Seq ID No	Cureon number/	Length and design	Sequence (Capital letters is β-D-oxy-LNA, s is phosphorothioate)
75	Cur2522*	16-mer fully thiolated	5'-$t_s c_s c_s g_s t_s c_s a_s t_s c_s g_s c_s t_s c_s c_s t_s$c-3'
76	Cur2524	16-mer LNA gapmer 3+3, fully thiolated	5'-$t_s C_s C_s G_s t_s c_s a_s t_s c_s g_s c_s t_s C_s C_s T_s$c-3'
77	Cur2525	16-mer, LNA gapmer 3+3, fully thiolated, 5 mismatches	5'-$t_s C_s \underline{A}_s G_s t_s \underline{a}_s a_s t_s \underline{a}_s g_s c_s \underline{c}_s C_s C_s \underline{A}_s$c-3'

* The benchmark oligonucleotide: ISIS 2503 n-4 i.e their oligonucleotide which is made 4 bp shorter.

Tumor growth analysis

Two separate experiments were carried out. Female NMRI nude mice of 7-8 weeks old were obtained from M&B. Mice were kept 5 in each cage and allowed to acclimatize at least one week before entering experiments. Mice were injected subcutaneous with 10^6 15PC3 human prostate cancer cells suspended in 300µl matrigel as previously described by K. Fleüter. One week after tumor cell injection the anti-HaRas oligonucleotides, the mismatch control oligo and PBS were administrated subcutaneously for 14 days using ALZET osmotic pumps (model 1002). Prior to dorsal implantation the pumps were incubated overnight at room temperature in sterile PBS. Each group included 5-6 mice.

Some mice carried two tumors. Tumor volumes were calculated according to the formula ($\pi \times L \times D^2/6$), where L represents the largest diameter and D the tumor diameter perpendicular to L. Each tumor was regarded as one experimental unit. The experiments were blinded. After end treatment (14 days) mice were sacrificed and tumors were excised, freezed and kept for protein analysis. Tumors weights were also recorded.

Results

Tumor growth was almost inhibited by the fully thiolated 16-mer LNA gapmer containing 3 LNA's in each flank (Cur2524). This effect was demonstrated at 2.5 mg/kg/day (Figure 3). The mismatch control oligonucleotide containing 5 bp mismatches (Cur2525) however did not have any anti-tumor effect. This demonstrated *in vivo* specificity of the LNA-containing antisense oligonucleotide (Cur2524) targeting Ha-ras. The anti-tumor effect of Cur2524 (LNA-gapmer) was compared with the 16-mer phosphorothioate (Cur2522). Inhibition of tumor growth by Cur2524 (LNA-gapmer) was demonstrated, while the iso-sequential 16-mer phosphorothioate had no effect (Figure 3).

Example 17 In vivo superiority of short LNA oligomeric compounds compared to longer phosphorthioate compound

ISIS 2503 is a well-known antisense oligonucleotide developed by ISIS pharmaceuticals that inhibits expression of Ha-Ras and that compound selected for clinical trials. This oligonucleotide has shown to inhibit tumour growth in several tumour xenograft models e.g. the 15PC3 xenografts (Fluiter et al. Cancer Res. 62, 2024-2028). The goal of this study was to compare the established ISIS 2503 with a LNA gapmer oligomeric compound that targets Ha-Ras in a nude mice model. A further goal was to investigate the potency of short (16-mer) LNA oligomeric compounds compared to a long phosphorothioate (20-mer).

Experimental design

The following oligonucleotides were synthesized. Cur 2119 is identical to ISIS2503. The oligonucleotides were fully thiolated. It is important to note that the LNA gapmers are 16mers while benchmark oligonucleotides are 20 mers. The compounds were checked

using MALDI-TOF analysis (data not shown). The compounds were sufficiently purified for use in the in vivo experiments.

Table 4 LNA compounds as 16-mers and benchmark phosphorothioate as 20-mer

Seq ID No	Cureon number	Length and design	Sequence (5'-3')
78	Cur 2119	DNA 20-mer	tccgtcatcgctcctcaggg
79	Cur 2131	β-D-oxy-LNA (captured letters)/DNA gapmer 16-mer	TCCGtcatcgctCCTC

In vivo tumor growth inhibition

Eight to ten week old NMRI nu/nu mice (Charles River, the Netherlands) were injected subcutaneously in the flank with 10^6 MiaPaca II cells or 10^6 15PC3 cells in 300 µl Matrigel (Collaborative Biomedical products, Bedford, Ma, USA). The cells were injected within one hour after harvesting by trypsine treatment. Before injection the cells were washed with cold PBS, counted with a haemocytometer and subsequently mixed with the Matrigel on ice. One week after tumor cell injection, when tumor take was positive, an osmotic mini pump (Alzet model 1002, lot. number10017-00, Alzet corp., Palo Alto, Ca, USA) was implanted dorsally according to the instructions of the manufacturer. The osmotic minipumps were incubated in PBS 20 hours at 37°C prior to implantation to start up the pump. The osmotic minipumps were filled with oligonucleotides (1 mg/kg/day) or 0.9% saline. Tumor growth was monitored daily following the implantation of the osmotic mini pump. Tumor volume was measured and calculated as described previously (Meyer, et al. Int J. Cancer, 43: 851-856, 1989.). All mice were implanted with IPTT-200 temperature transponder chips (BMDS inc., Seaford, Delaware, USA) to allow temperature measurements and identification of the mice using a DAS 5002 scanner (BMDS inc.) during treatment.

Nude mice were injected s.c. with Miapaca II cells (right flank) and 15PC3 cells (left Flank) one week prior to the start of ODN treatment to allow xenograft growth. The anti Ha-Ras compounds (Cur 2119 and Cur 2131) and controls (Cur 2120 and Cur 2132) were administrated for 14 days using Alzet osmotic minipumps (model 1002) implanted

dorsally. Dosages used were 1mg/kg/day. During treatment the tumor growth was monitored.

It can be concluded that the 16mer LNA containing gapmer is more potent as the 20-mer phosphorthioate oligonucleotide (see Figure 4).

Example 18 In vivo potency of alpha-LNA oligomeric compounds are at least as good as the beta-D-oxy LNA oligomeric compounds

Nude mice were injected s.c. with MiaPaca II cells (right flank) and 15PC3 cells (left flank) one week prior to the start of oligonucleotide treatment to allow xenograft growth. The anti HaRas oligonucleotides (2713, 2722, 2742 and 2776) and control oligonucleotides (2744 and 2778) (see table 5) were administrated for 14 days using Alzet osmotic minipumps (model 1002) implanted dorsally. Three dosages were used: 1, 2.5 and 5 mg/Kg/day for all of them, except for 2722 and 2713, for which a dosage of 5 mg/Kg/day was administered. During treatment the tumor growth was monitored. Tumor growth was almost inhibited completely at 5 mg/Kg/day, 2.5 mg/Kg/day and even at 1 mg/Kg/day dose with 2742 and 2776 in 15PC3 cells, figure 8. The specificity with control oligonucleotides (2744 and 2778, containing mismatches) increased as the dose decreased. At 1 mg/Kg/day dose the experiment presented a good specificity, particularly for alpha-L-oxy-LNA oligonucleotides (2742 and 2744). In MiaPacaII xenograft tumors, the effect of the oligonucleotides is in general comparable with those on the 15PC3 xenografts, except for the fact that the specificity seemed to be a bit lower. For 2713 and 2722, a potent inhibition of tumor growth was also observed, see figure 9. It can be concluded that the oligonucleotide containing alpha-L-oxy-LNA are as potent, or maybe even better, as the one containing beta-D-oxy-LNA in tumor growth inhibition in the concentration range tested.

Table 5. Oligonucleotides containing alpha-L-oxy-LNA and beta-D-oxy-LNA used in the in vivo experiment. Residue c is methyl-c both for DNA and LNA, except for c DNA in 2713 and 2722.

ref	oligonucleotides	
2776	$T^{\alpha}_{s}C^{\alpha}_{s}C^{\alpha}_{s}g_{s}t_{s}c_{s}a_{s}t_{s}c_{s}g_{s}c_{s}t_{s}C^{\alpha}_{s}C^{\alpha}_{s}T^{\alpha}_{s}c$	match
2778	$T^{\alpha}_{s}C^{\alpha}_{s}T^{\alpha}_{s}g_{s}t_{s}a_{s}a_{s}t_{s}a_{s}g_{s}c_{s}c_{s}C^{\alpha}_{s}C^{\alpha}_{s}C^{\alpha}_{s}c$	Mismatch control
2742	$T_{s}C_{s}C_{s}g_{s}t_{s}c_{s}a_{s}t_{s}c_{s}g_{s}c_{s}t_{s}C_{s}C_{s}T_{s}c$	match
2744	$T_{s}C_{s}T_{s}g_{s}t_{s}a_{s}a_{s}t_{s}a_{s}g_{s}c_{s}c_{s}C_{s}C_{s}C_{s}c$	Mismatch control
2713	$C_{s}C_{s}C_{s}A_{s}t_{s}c_{s}t_{s}g_{s}t_{s}g_{s}c_{s}c_{s}C_{s}G_{s}A_{s}C$	Match
2722	$T_{s}C_{s}C_{s}T_{s}c_{s}c_{s}t_{s}t_{s}c_{s}c_{s}g_{s}t_{s}t_{s}C_{s}T_{s}G_{s}C$	Match

Example 19 alpha-L-oxy-LNA and beta-D-oxy-LNA targeting Ha-ras show low toxicity levels in mice

The levels of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase in the serum were determined, in order to study the possible effects of this 14-day treatment in the nude mice. Serum samples were taken from each mouse after the 14-day experiment. From figure 10, ALAT levels in the serum varied between 250-500 U/L. ASAT levels were in the range of 80-150 U/L. The mice did not seem externally to be sick, and no big changes in behavior were observed. During treatment the body temperature of the mice was also monitored using IPTT-200 temperature transponders (figure 10). The body temperature did not change significantly during the treatment, not even at high dose 5 mg/Kg/day, which is an indication that no major toxicity effects are occurring.

The present invention has been described with specificity in accordance with certain of its preferred embodiments. Therefore, the following examples serve only to illustrate the invention and are not intended to limit the same.

Claims

What is claimed is:

1. A compound of 8 to 50 nucleobases in length which modulates *ras* and wherein said compound has a sequence comprising at least an 8-nucleobase portion of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77 or 79.

2. A compound of according to claim 1, in which the modulation is a ras inhibition and the ras is selected from Ha-ras, Ki-ras or N-ras.

3. A compound of 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding Ha-ras, wherein said compound specifically hybridises with a nucleic acid encoding Ha-ras and inhibits the expression of Ha-ras and wherein said compound has a sequence comprising at least an 8-nucleobase portion of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77 or 79.

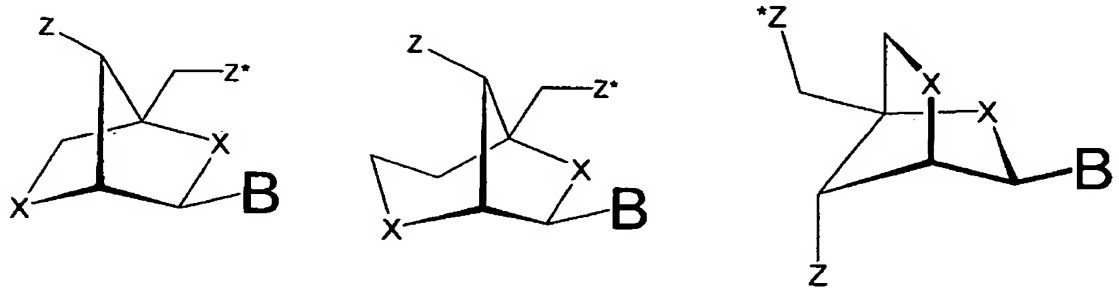
4. The compound according to any of claims 1-3, which is an antisense oligonucleotide.

5. The compound according to any of claims 1-4, wherein at least one nucleotide comprises at least one Nucleic Acid Analogue.

6. The compound according to any of claims 1-5, wherein at least one nucleotide comprises at least one Locked Nucleic Acid (LNA).

7. The compound according to claims 6, wherein the Locked Nucleic Acid (LNA) has the structure of the general formula in Scheme 2.

8. The compound according to claim 6, wherein at least one nucleotide comprises a Locked Nucleic Acid (LNA) unit according any of the formulas in Scheme 3:



Scheme 3

Where X is independently selected from -O-, -S-, -NH-, and $N(R^H)$,
Z and Z* are independently selected from the group consisting of an internucleoside linkage, a terminal group or a protecting group,
B constitutes a natural or non-natural nucleobase.

9. The compound according to claim 6 or 7, wherein the internucleoside linkage may be selected from the group consisting of $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-S-P(O)_2-O-$, $-S-P(O,S)-O-$, $-S-P(S)_2-O-$, $-O-P(O)_2-S-$, $-O-P(O,S)-S-$, $-S-P(O)_2-S-$, $-O-PO(R^H)-O-$, $O-PO(OCH_3)-O-$, $-O-PO(NR^H)-O-$, $-O-PO(OCH_2CH_2S-R)-O-$, $-O-PO(BH_3)-O-$, $-O-PO(NHR^H)-O-$, $-O-P(O)_2-NR^H-$, $-NR^H-P(O)_2-O-$, $-NR^H-CO-O-$, where R^H is selected form hydrogen and C_{1-4}-alkyl.

10. The compound according to claim 5, 6 or 7, wherein the nucleobases is a modified nucleobases selected from the group consisting of 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 2-chloro-6-aminopurine.

11. The compound according to any of claims 5-8, wherein the LNA is oxy-LNA, thio-LNA, amino-LNA in either the D-ß or L-α configurations or combinations thereof.

12. A compound of 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding Ha-ras, wherein said compound specifically hybridises with a nucleic acid encoding Ha-ras and inhibits the expression of Ha-ras and wherein said compound has a sequence comprising at least an 8-nucleobase portion of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 76, 77 or 79.

13. The compound according to any of claims 1-10, wherein the antisense oligonucleotide is a design according to any of the designs presented in Figure 1.

14. The compound according to claim 12, wherein the antisense oligonucleotide is a gapmer.

15. The compound according to any of the claims 1-14, wherein the antisense oligonucleotide is a 13, 14, 15, 16, 17, 18, 19, 20 or 21-mer.

16. The compound according to any of the claims 1-15, wherein the number of LNA units are 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21.

17. A pharmaceutical composition comprising the compound of any of claims 1-16, which further comprises a pharmaceutically acceptable carrier.

18. A pharmaceutical composition comprising the compound of any of claims 1-16, which is employed in a pharmaceutically acceptable salt.

19. A pharmaceutical composition comprising the compound of any of claims 1-16, which further comprises a conjugate or formulation.

20. A pharmaceutical composition comprising the compound of any of claims 1-16, which is constitutes a pro-drug.

21. A pharmaceutical composition comprising the compound of any of claims 1-16, which further comprises other oligomeric compounds, chemotherapeutic compounds, antiinflamatory compounds and/or antiviral compounds.

22. A method of inhibiting the expression of Ha-ras, in cells or tissues comprising contacting said cells or tissues with the compound according to any of claims 1-16 so that expression of Ha-ras is inhibited.

23. A method of modulating expression of a gene involved in a cancer disease comprising contacting the gene or RNA from the gene with an oligomeric compound wherein said compound has a sequence comprising at least an 8 nucleobase portion of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77 or 79 whereby gene expression is modulated.

24. A method according to claim 23, wherein the compounds comprises one or more LNA units.

25. The method of claim 23 or 24, wherein the compound hybridizes with messenger RNA of the gene to inhibit expression thereof.

26. A method of treating a mammal suffering from or susceptible from an cancer disease, comprising:
administering to the mammal an therapeutically effective amount of an oligonucleotide targeted to Ha-ras that comprises one or more LNA units.

27. The method according to any of the claims 23-26, wherein the cancer diseases is a lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries cancer.

28. A method of modulating the red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation or matrix metabolism comprising contacting a cell with the antisense compound of claim 1-16 so that the cell is modulated.

29. A method of inhibiting the proliferation of cells comprising contacting cells in vitro with an effective amount of the antisense compound of claim 1-16, so that proliferation of the cells is inhibited.

30. The method of claim 29 wherein said cells are cancer cells.

ABSTRACT

Oligonucleotides directed against the Ha-ras gene are provided for modulating the expression of Ha-ras. The compositions comprise oligonucleotides, particularly antisense oligonucleotides, targeted to nucleic acids encoding the Ha-ras. Methods of using these compounds for modulation of Ha-ras expression and for the treatment of diseases associated with either overexpression of Ha-ras, expression of mutated Ha-ras or both are provided. Examples of diseases are cancer such as lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries cancers. The oligonucleotides may be composed of deoxyribonucleosides or a nucleic acid analogue such as for example locked nucleic acid or a combination thereof.

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Figure 1/10

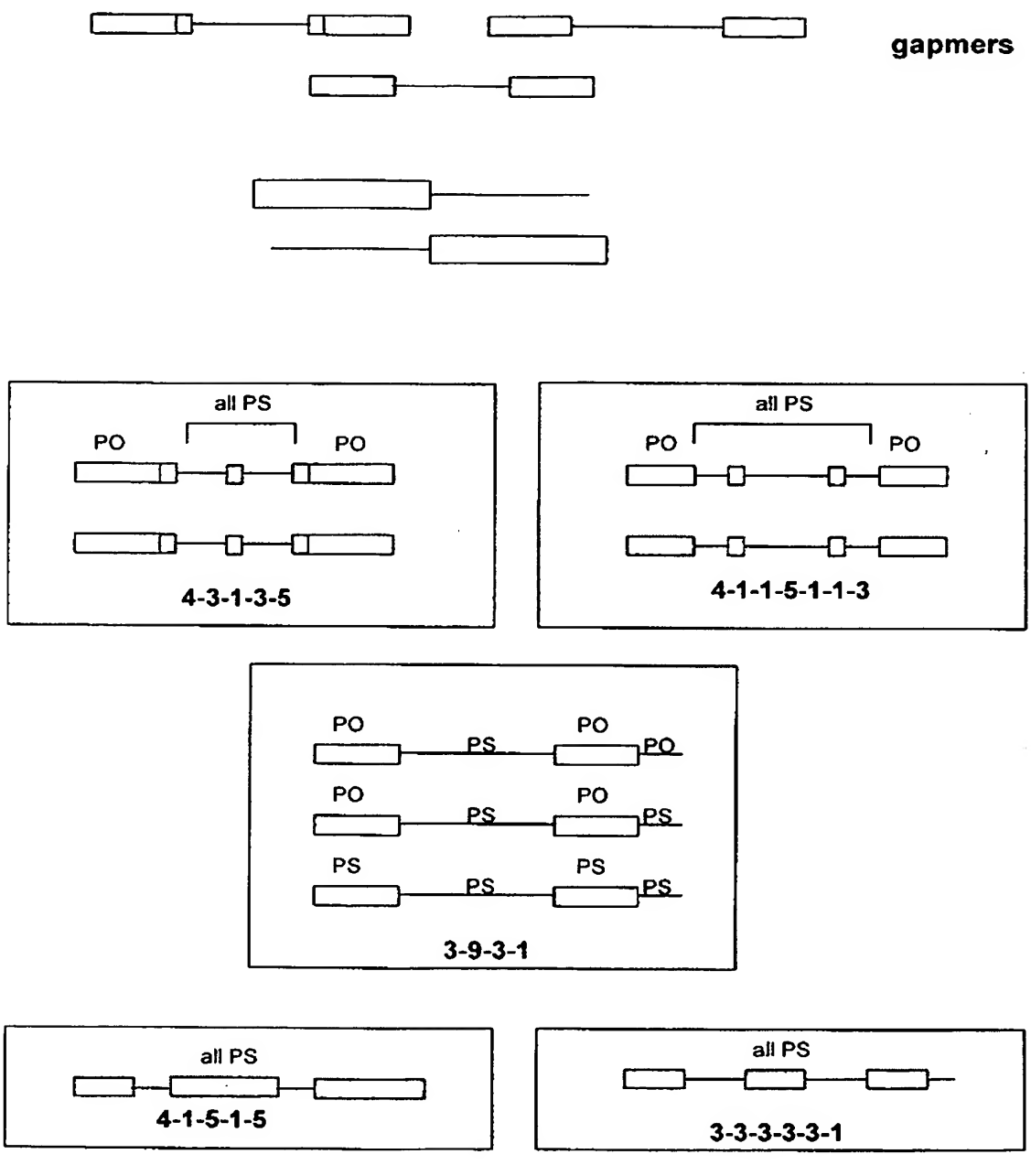
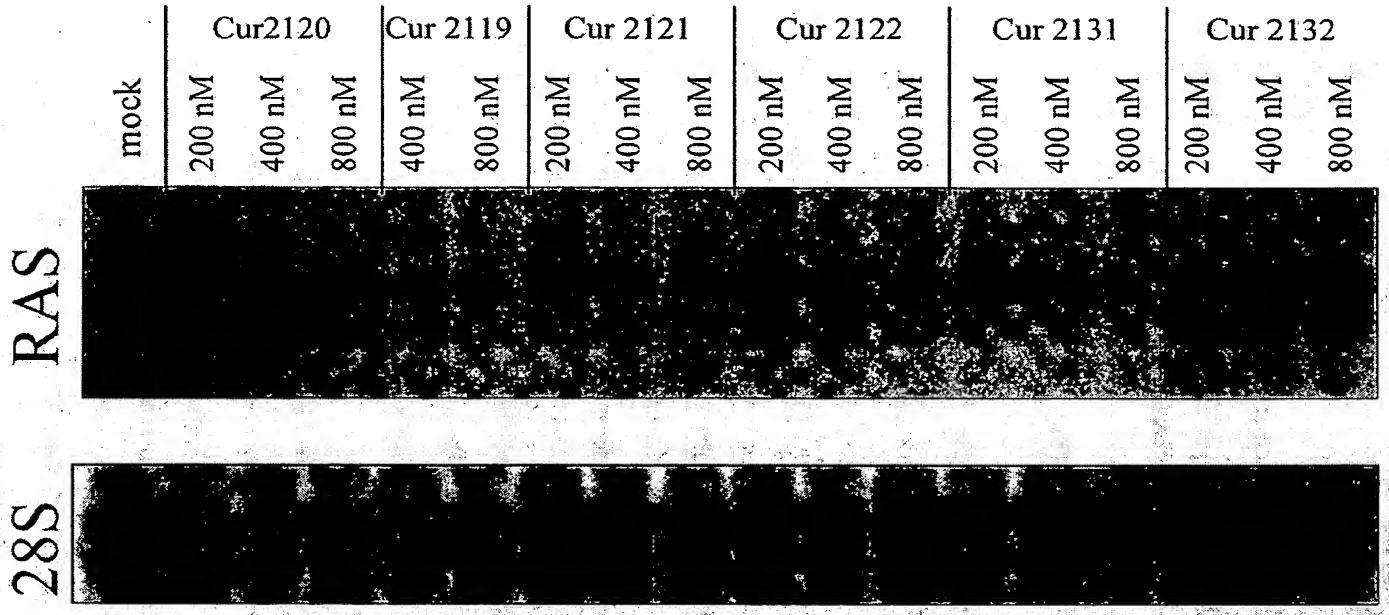


Figure 2/10



% mRNA relative to mock		**200nM**	**400nM**	**800nM**
Cur2119	PS20	-	35 (27)	21 (12)
Cur2120	PS20x6mm	80 (62)	83 (63)	53 (40)
Cur2121	LNA20	57 (43)	45 (30)	42 (26)
Cur2122	LNA20x6mm	88 (87)	65 (66)	52 (50)
Cur2131	LNA16	45 (44)	30 (26)	*31* (16)
Cur2132	LNA16x6mm	*145* (79)	*119* (76)	*110* (71)

Figure 3/10

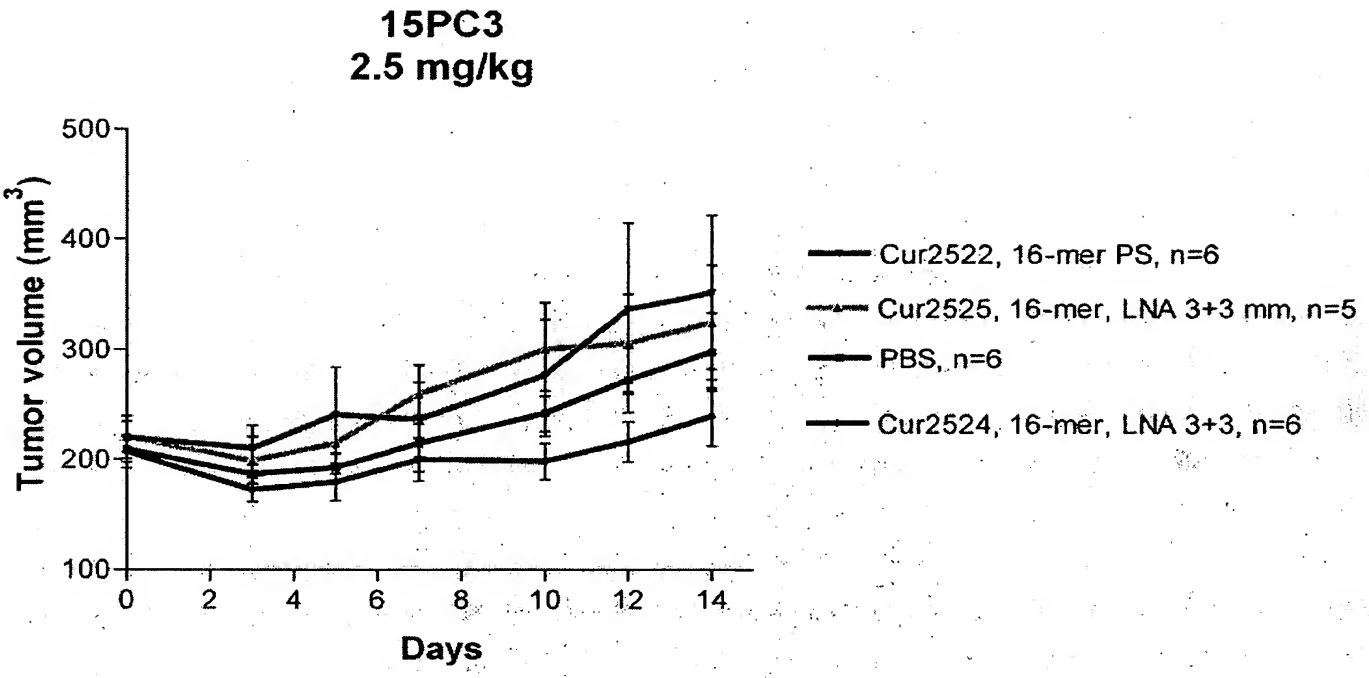


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Figure 4/10

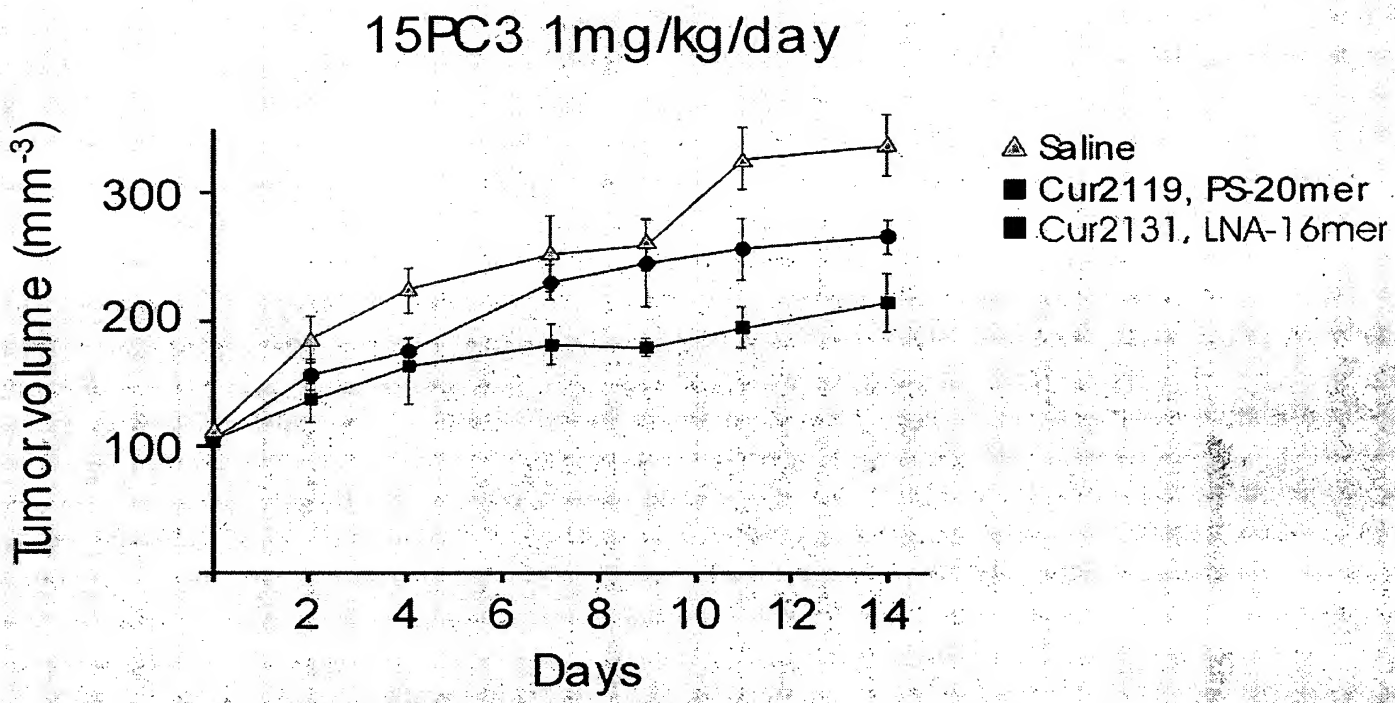

Figure 5/10

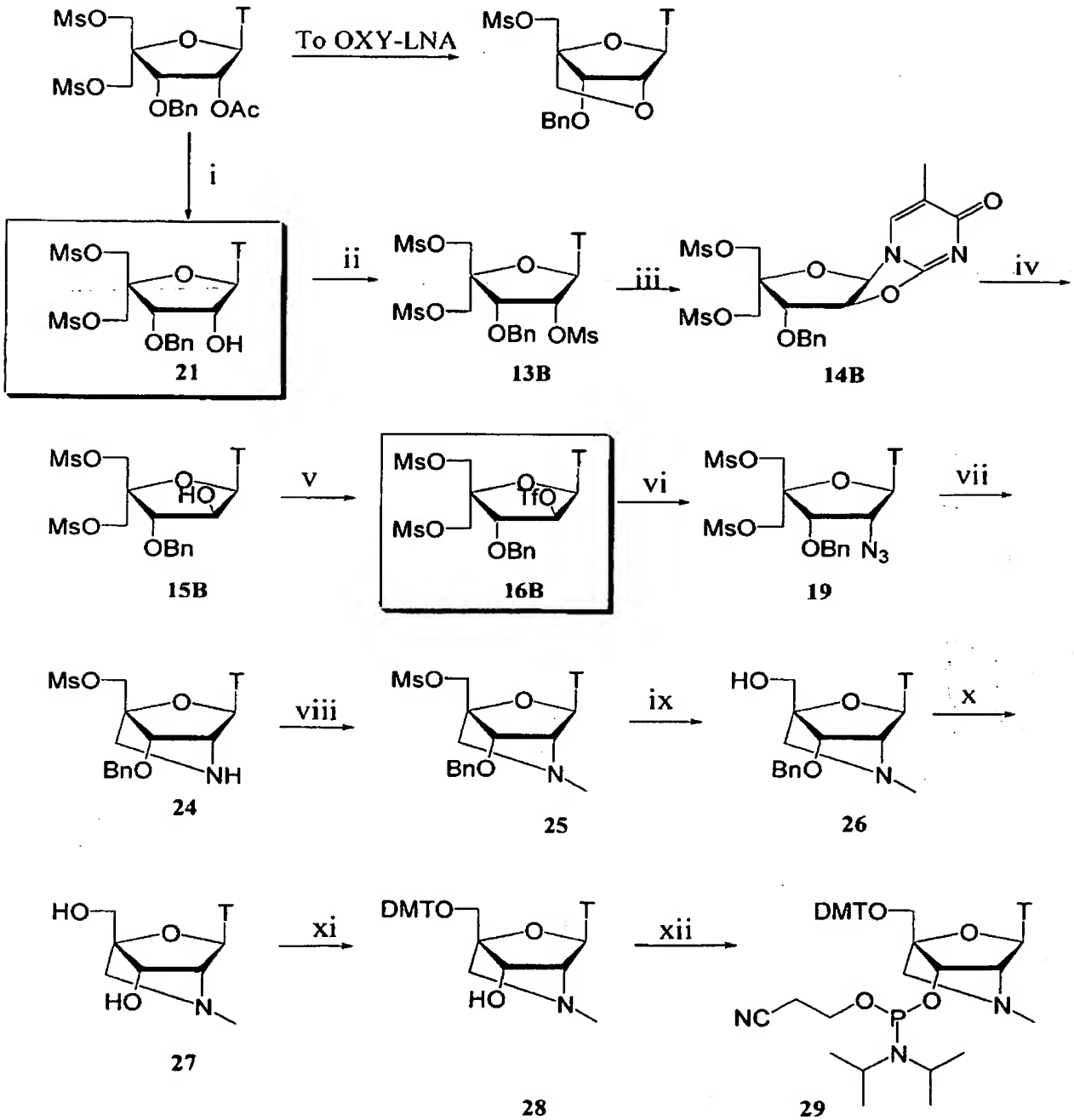

Figure 6/10

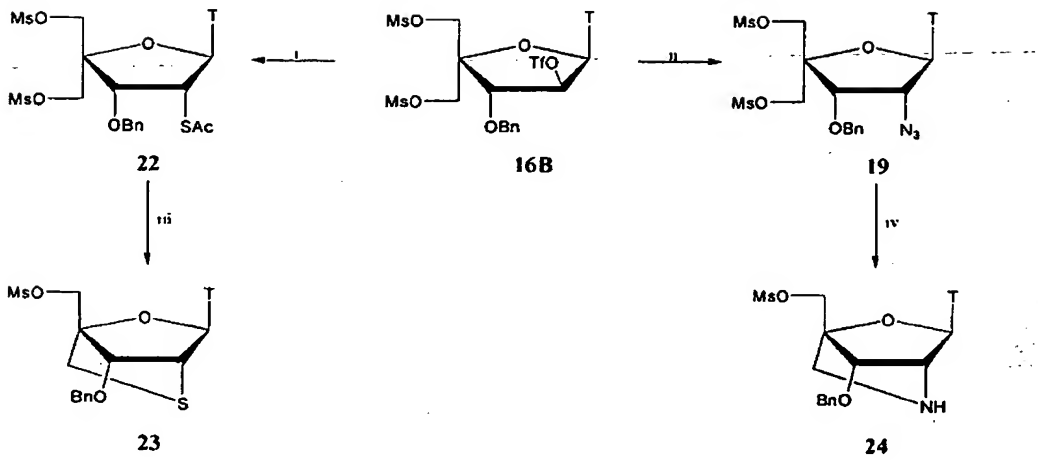

Figure 7/10

Seq ID NO: 1

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      CCTAGGGTCG GAAAGGGGTC GGGCATCGGG GCCCTGGAGG CGCCACCCGC
  51  GCGCCGCGCT GCCGGCGCAG GGAGGGCCTC TGGTGCACCG GCACCGCTGA
      CGCGGCGCGA CGGCCGCGTC CCTCCCGGAG ACCACGTGGC CGTGGCGACT
 101  GTCGGGTTCT CTCGCCGGCC TGTTCCCGGG AGAGCCCGGG GCCCTGCTCG
      CAGCCCAAGA GAGCGGCCGG ACAAGGGCCC TCTCGGGCCC CGGGACGAGC
 151  GAGATGCCGC CCCGGGCCCC CAGACACCGG CTCCCTGGCC TTCCTCGAGC
      CTCTACGGCG GGGCCCGGGG GTCTGTGGCC GAGGGACCGG AAGGAGCTCG
 201  AACCCCGAGC TCGGCTCCGG TCTCCAGCCA AGCCCAACCC CGAGAGGCCG
      TTGGGGCTCG AGCCGAGGCC AGAGGTCGGT TCGGGTTGGG GCTCTCCGGC
 251  CGGCCCTACT GGCTCCGCCT CCCGCGTTGC TCCCGGAAGC CCCGCCCGAC
      GCCGGGATGA CCGAGGCGGA GGGCGCAACG AGGGCCTTCG GGGCGGGCTG
 301  CGCGGCTCCT GACAGACGGG CCGCTCAGCC AACCGGGGTG GGGCGGGGCC
      GCGCCGAGGA CTGTCTGCCC GGCGAGTCGG TTGGCCCCAC CCCGCCCCGG
 351  CGATGGCGCG CAGCCAATGG TAGGCCGCGC CTGGCAGACG GACGGGCGCG
      GCTACCGCGC GTCGGTTACC ATCCGGCGCG GACCGTCTGC CTGCCCGCGC
 401  GGGCGGGGCG TGCGCAGGCC CGCCCGAGTC TCCGCCGCCC GTGCCCTGCG
      CCCGCCCCGC ACGCGTCCGG GCGGGCTCAG AGGCGGCGGG CACGGGACGC
 451  CCCGCAACCC GAGCCGCACC CGCCGCGGAC GGAGCCCATG CGCGGGGCGA
      GGGCGTTGGG CTCGGCGTGG GCGGCGCCTG CCTCGGGTAC GCGCCCCGCT
 501  ACCGCGCGCC CCCGCCCCCG CCCCGCCCCG GCCTCGGCCC CGGCCCTGGC
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 551  CCCGGGGGCA GTCGCGCCTG TGAACGGTGA GTGCGGGCAG GGATCGGCCG
      GGGCCCCCGT CAGCGCGGAC ACTTGCCACT CACGCCCGTC CCTAGCCGGC
 601  GGCCGCGCGC CCTCCTCGCC CCCAGGCGGC AGCAATACGC GCGGCGCGGG
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 651  CCGGGGGCGC GGGGCCGGCG GGCGTAAGCG GCGGCGGCGG CGGCGGGTGG
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      CACCCCGGCC CGCCCCGGGC GCCCGTGTCC ACTCGCCCGC AGCCCCCGAC
 751  CGGCGGGCGG GGGCCCCTTC CTCCCTGGGG CCTGCGGGAA TCCGGGCCCC
      GCCGCCCGCC CCCGGGGAAG GAGGGACCCC GGACGCCCTT AGGCCCGGGG
 801  ACCCGTGGCC TCGCGCTGGG CACGGTCCCC ACGCCGGCGT ACCCGGGAGC
      TGGGCACCGG AGCGCGACCC GTGCCAGGGG TGCGGCCGCA TGGGCCCTCG
 851  CTCGGGCCCG GCGCCCTCAC ACCCGGGGGC GTCTGGGAGG AGGCGGCCGC
      GAGCCCGGGC CGCGGGAGTG TGGGCCCCCG CAGACCCTCC TCCGCCGGCG
 901  GGCCACGGCA CGCCCGGGCA CCCCCGATTC AGCATCACAG GTCGCGGACC
      CCGGTGCCGT GCGGGCCCGT GGGGGCTAAG TCGTAGTGTC CAGCGCCTGG
 951  AGGCCGGGGG CCTCAGCCCC AGTGCCTTTT CCCTCTCCGG GTCTCCCGCG
      TCCGGCCCCC GGAGTCGGGG TCACGGAAAA GGGAGAGGCC CAGAGGGCGC
1001  CCGCTTCTCG GCCCCTTCCT GTCGCTCAGT CCCTGCTTCC CAGGAGCTCC
      GGCGAAGAGC CGGGGAAGGA CAGCGAGTCA GGGACGAAGG GTCCTCGAGG
1051  TCTGTCTTCT CCAGCTTTCT GTGGCTGAAA GATGCCCCCG GTTCCCCGCC
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1101  GGGGGTGCGG GGCGCTGCCC GGGTCTGCCC TCCCCTCGGC GGCGCCTAGT
      CCCCCACGCC CCGCGACGGG CCCAGACGGG AGGGGAGCCG CCGCGGATCA
1151  ACGCAGTAGG CGCTCAGCAA ATACTTGTCG GAGGCACCAG CGCCGCGGGG
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1201  CCTGCAGGCT GGCACTAGCC TGCCCGGGCA CGCCGTGGCG CGCTCCGCCG
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      GGACGTCCGA CCGTGATCGG ACGGGCCCGT GCGGCACCGC GCGAGGCGGC
1251  TGGCCAGACC TGTTCTGGAG GACGGTAACC TCAGCCCTCG GGCGCCTCCC
      ACCGGTCTGG ACAAGACCTC CTGCCATTGG AGTCGGGAGC CCGCGGAGGG
1301  TTTAGCCTTT CTGCCGACCC AGCAGCTTCT AATTTGGGTG CGTGGTTGAG
      AAATCGGAAA GACGGCTGGG TCGTCGAAGA TTAAACCCAC GCACCAACTC
1351  AGCGCTCAGC TGTCAGCCCT GCCTTTGAGG GCTGGGTCCC TTTTCCCATC
      TCGCGAGTCG ACAGTCGGGA CGGAAACTCC CGACCCAGGG AAAAGGGTAG
1401  ACTGGGTCAT TAAGAGCAAG TGGGGGCGAG GCGACAGCCC TCCCGCACGC
      TGACCCAGTA ATTCTCGTTC ACCCCCGCTC CGCTGTCGGG AGGGCGTGCG
1451  TGGGTTGCAG CTGCACAGGT AGGCACGCTG CAGTCCTTGC TGCCTGGCGT
      ACCCAACGTC GACGTGTCCA TCCGTGCGAC GTCAGGAACG ACGGACCGCA
1501  TGGGGCCCAG GGACCGCTGT GGGTTTGCCC TTCAGATGGC CCTGCCAGCA
      ACCCCGGGTC CCTGGCGACA CCCAAACGGG AAGTCTACCG GGACGGTCGT
1551  GCTGCCCTGT GGGGCCTGGG GCTGGGCCTG GGCCTGGCTG AGCAGGGCCC
      CGACGGGACA CCCCGGACCC CGACCCGGAC CCGGACCGAC TCGTCCCGGG
1601  TCCTTGGCAG GTGGGGCAGG AGACCCTGTA GGAGGACCCC GGGCCGCAGG
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1651  CCCCTGAGGA GCGATGACGG AATATAAGCT GGTGGTGGTG GGCGCCGGCG
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1701  GTGTGGGCAA GAGTGCGCTG ACCATCCAGC TGATCCAGAA CCATTTTGTG
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1751  GACGAATACG ACCCCACTAT AGAGGTGAGC CTAGCGCCGC CGTCCAGGTG
      CTGCTTATGC TGGGGTGATA TCTCCACTCG GATCGCGGCG GCAGGTCCAC
1801  CCAGCAGCTG CTGCGGGCGA GCCCAGGACA CAGCCAGGAT AGGGCTGGCT
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1851  GCAGCCCCTG GTCCCCTGCA TGGTGCTGTG GCCCTGTCTC CTGCTTCCTC
      CGTCGGGGAC CAGGGGACGT ACCACGACAC CGGGACAGAG GACGAAGGAG
1901  TAGAGGAGGG GAGTCCCTCG TCTCAGCACC CCAGGAGAGG AGGGGGCATG
      ATCTCCTCCC CTCAGGGAGC AGAGTCGTGG GGTCCTCTCC TCCCCCGTAC
1951  AGGGGCATGA GAGGTACCAG GGAGAGGCTG GCTGTGTGAA CTCCCCCCAC
      TCCCCGTACT CTCCATGGTC CCTCTCCGAC CGACACACTT GAGGGGGGTG
2001  GGAAGGTCCT GAGGGGGTCC CTGAGCCCTG TCCTCCTGCA GGATTCCTAC
      CCTTCCAGGA CTCCCCCAGG GACTCGGGAC AGGAGGACGT CCTAAGGATG
2051  CGGAAGCAGG TGGTCATTGA TGGGGAGACG TGCCTGTTGG ACATCCTGGA
      GCCTTCGTCC ACCAGTAACT ACCCCTCTGC ACGGACAACC TGTAGGACCT
2101  TACCGCCGGC CAGGAGGAGT ACAGCGCCAT GCGGGACCAG TACATGCGCA
      ATGGCGGCCG GTCCTCCTCA TGTCGCGGTA CGCCCTGGTC ATGTACGCGT
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2301  TCCTCTCTGC GCATGTCCTG GATGCCGCTG CGCCTGCAGC CCCCGTAGCC
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2351  AGCTCTCGCT TTCCACCTCT CAGGGAGCAG ATCAAACGGG TGAAGGACTC
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2551  CCCACAGCTA GCCAGGGACC CGCCCCGCCC CGCCCCAGCC AGGGAGCAGC
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      TGAGTGACTG GGAGAGGGAA CTGTGTCCCG TCGGCGAGAC CGAGATCGAG
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4651   CCCTCTGGGA CGGGGACTTG TGCCCTGTCA GGGTTCCCTA TCCCTGAGGT
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        CTCGGGAGAT CCCGACGGCC CACGGTGGGA CCGAGGAAGG TGTGGCACGA
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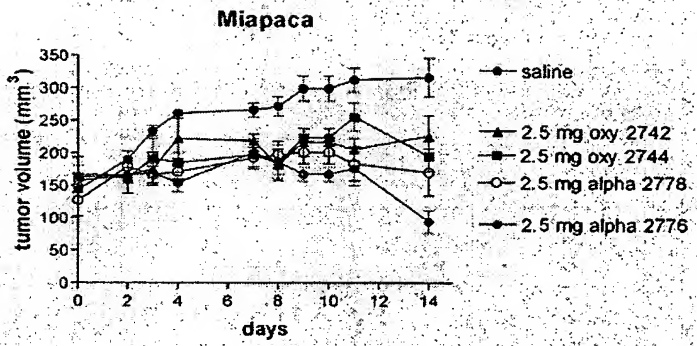
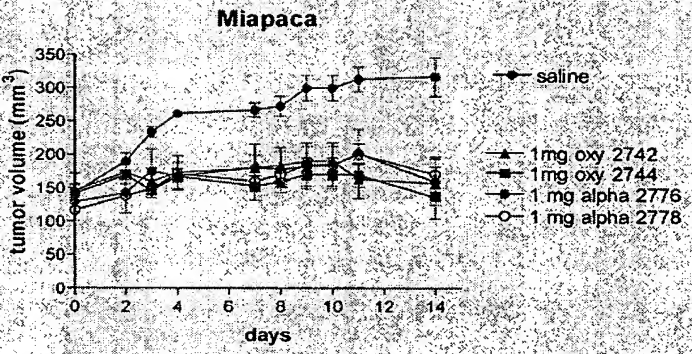
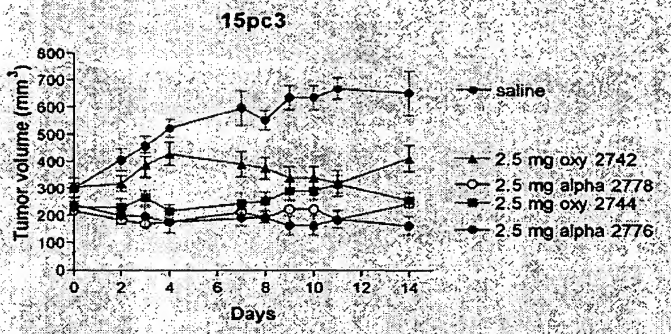
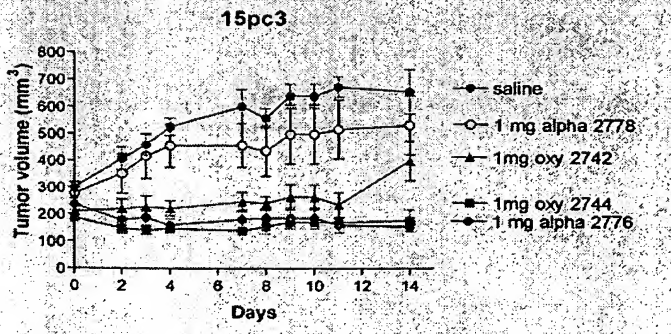
Figure 8/10









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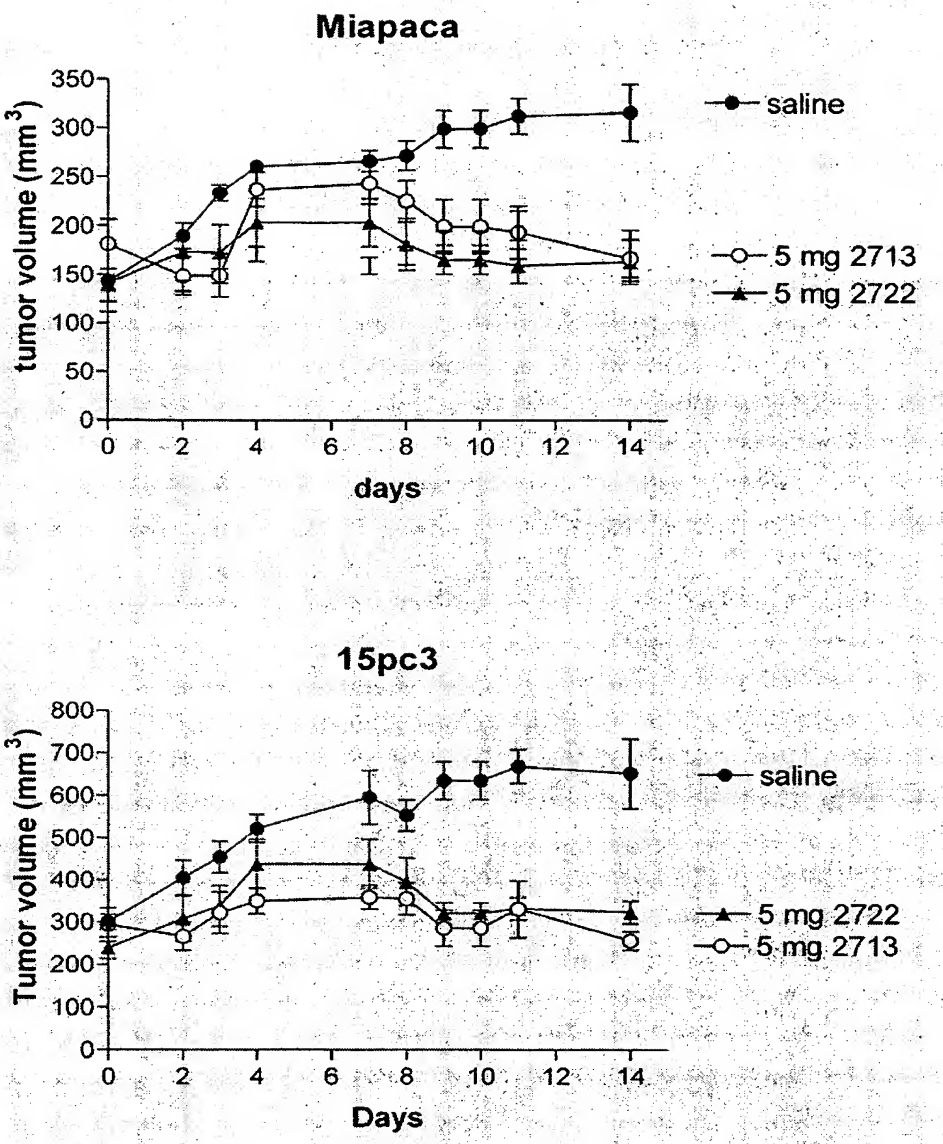

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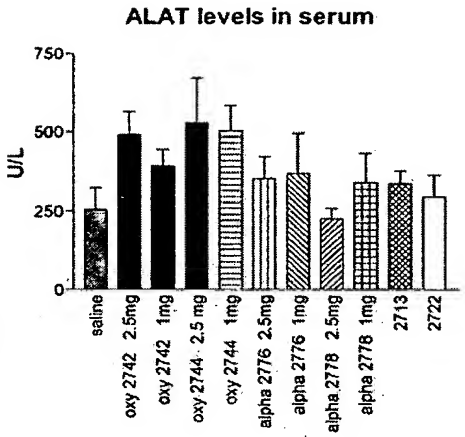

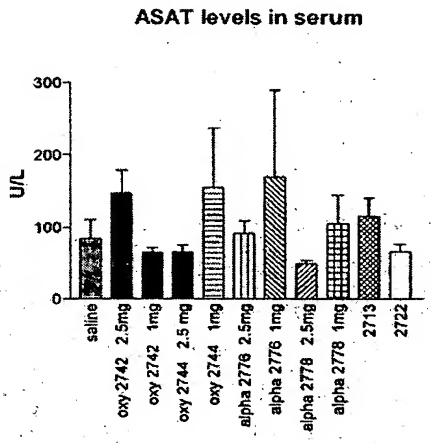

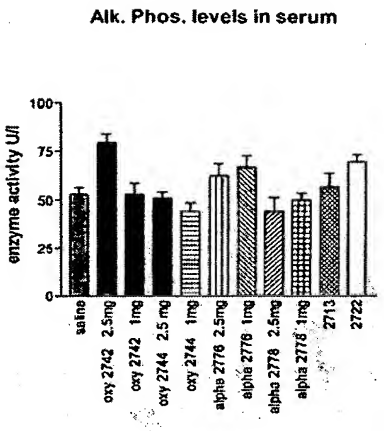

Group 1	saline	
Group 2	2.5 mg	2742
Group 3	1 mg	2742
Group 4	2.5 mg	2744
Group 5	1 mg	2744
Group 6	2.5 mg	2776
Group 7	1 mg	2776
Group 8	2.5 mg	2778
Group 9	1 mg	2778
Group 10	5 mg	2713
Group 11	5 mg	2722